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Alterations in Synaptic Strength Preceding Axon Withdrawal

H. Colman, J. Nabekura,* J. W. Lichtman†

Permanent removal of axonal input to postsynaptic cells helps shape the pattern of neuronal connections in response to experience, but the process is poorly understood. Intracellular recording from newborn and adult mouse muscle fibers temporarily innervated by two axons showed an increasing disparity in the synaptic strengths of the two inputs before one was eliminated. The connection that survived gained strength by increasing the amount of neurotransmitter released (quantal content), whereas the input that was subsequently removed became progressively weaker, because of a reduction in quantal content and a reduction in quantal efficacy associated with reduced postsynaptic receptor density. Once the synaptic strengths of two inputs began to diverge, complete axonal withdrawal of the weaker input occurred within 1 to 2 days. These experiments provide a link between experience-driven changes in synaptic strength and long-term changes in connectivity in the mammalian nervous system.

The ability of the nervous system to respond to experience in an enduring way may depend on alterations in the structure or function of synaptic connections. An indelible synaptic alteration induced by experience early in postnatal life is the loss of some of the axonal inputs that converge on a target cell (*1*). A large body of work also indicates that the strength of existing synapses can be potentiated or depressed in response to activity (*2*), and several investigations have suggested that such changes in synaptic efficacy ultimately lead to structural plasticity (*3*). However, the relation between alterations in synaptic strength and permanent structural changes in synaptic connectivity is not well understood.

The neuromuscular junction is a simple and accessible place to examine the relation between functional and structural synaptic changes, especially during early postnatal life when each muscle fiber undergoes a transition from polyneuronal to single innervation (*4*). Earlier attempts with the use of techniques with relatively low sensitivity failed to detect functional correlates of synapse loss at the neuromuscular junction, and led to the hypothesis that the loss of synaptic transmission during synapse elimination must be abrupt and must result from the sudden degeneration of the eliminated axonal branch and all of its synapses (*5*). Subsequent anatomical studies, however, provided no evidence of degeneration (*6*) and later studies demonstrated a progressive loss of synaptic area before axon withdrawal (*7*). Utilizing more sensitive physiological techniques, we have now reexamined whether alterations in synaptic strength occur prior to axonal withdrawal.

Intracellular recording from muscle fibers (n = 600) in the mouse trapezius muscle, chosen because of its favorable anatomical features, showed that most fibers underwent the transition from multiple to single axonal innervation during the first two postnatal weeks with some muscle fibers achieving single innervation substantially earlier than others (*8*). On postnatal day 2, approximately three-fourths of muscle fibers were multiply innervated (>95 percent by two axons), whereas about one-third were multiply innervated on day 6 and less than one-tenth remained multiply innervated at day 10. The progressive loss of polyneuronal innervation in the trapezius indicated that synapse elimination here (as elsewhere) was not occurring synchronously on each postsynaptic cell. Rather, some target cells achieved single innervation much sooner than others. Thus, if there were functional changes in synaptic strength associated with the elimination of synapses, at any one time, different muscle fibers should be at different stages in this process.

In order to independently activate two axons converging at the same junction, suction electrodes were applied to two nearby nerve branches projecting to the same region of the neonatal mouse trapezius (Fig. 1A). Muscle fibers innervated by two axons (Fig. 1B), one input traveling through each of these nerve branches, were selected for study by intracellular recording. High magnesium (10 to 17 mM) recording solution was used to reduce the size of the evoked endplate potentials (EPPs) (Fig. 1C) (*9*), so that the quantal content of each input could be measured by repetitive stimulation (mean = 770) with the method of failures (*10*).

Changes in quantal content. During the first 10 days after birth, the average quantal contents of the inputs to individual fibers diverged (Fig. 2A). At young ages (P1 to

The authors are in the Department of Anatomy and Neurobiology, Box 8108, Washington University School of Medicine, 660 South Euclid, St. Louis, MO 63110, USA.

*Present address: Department of Physiology, Kyushu University Faculty of Medicine, 3-1-1 Maidashi Higashi-ku, Fukuoka, 812-82 Japan.

†To whom correspondence should be addressed.

P3), when most fibers were still multiply innervated, the majority were contacted by two axons whose synaptic strengths were relatively similar (quantal content ratios <2) (Fig. 2B). At older ages, as more fibers became singly innervated, the strengths of the inputs to the remaining multiply innervated fibers shifted. At P4 to P6, the moderately large quantal content ratios (2 to 4) occurred most often, whereas at P7 to P9 the largest ratios (>4) predominated in the few fibers that were still multiply innervated. Furthermore, the absolute number of fibers having two inputs with very large differences in quantal content (that is, with ratios greater than 4) was doubled during the first postnatal week, at a time when the number of fibers having two inputs with smaller differences decreased. Therefore, muscle fibers that at early ages had inputs with similar quantal contents must have given rise to the fibers observed at later ages in which the quantal contents were more different.

The synaptic strengths of each of the two inputs to multiply innervated fibers were compared with the synaptic strength of the one input remaining on singly innervated fibers in the same muscle to learn whether it was the weaker or stronger input that was eliminated. At each age, the singly innervated muscle fibers had quantal contents that were on average most similar to the stronger input to multiply innervated fibers having the widest disparities in quantal content (ratios greater than 4) (Fig. 2C). This result suggests that during the competitive process that leads to synapse elimination the axon that is weaker is eliminated and the stronger input is preferentially maintained. Moreover, it suggests that muscle fibers receiving inputs with the widest disparities in quantal content (quantal content ratio greater than 4) (Fig. 2C) are the direct predecessors to singly innervated muscle fibers.

The increasing disparity in quantal content of the inputs co-innervating a muscle fiber was due both to an absolute increase in the quantal content of the stronger input and an absolute decrease in the quantal content of the weaker input ($P < 0.01$ for both, one-way ANOVA) (Fig. 2D). The changes in quantal content may be related to a growing disparity in synaptic area occupied by each axon (*7*) because synaptic area correlates with quantity of neurotransmitter released (*11*). Thus, axon withdrawal is preceded by changes in both the structure and function of an axon.

Changes in quantal efficacy. The use of high magnesium also permitted measurement of the depolarization induced by individual quanta (quantal efficacy) for each input to a muscle fiber. Alterations in quantal size were observed that were distinct from the changes in quantal content. Ranking the synaptic responses from each input independently by amplitude and displaying the ranked synaptic potentials as individual horizontal lines in an image (Fig. 3, A to D) showed that in approximately 30 percent (37/124) of multiply innervated muscle fibers, one input gave rise to quantal responses that were significantly smaller than those from the other innervating axon (*12*).

An example of a multiply innervated fiber having one input (axon 1) with reduced quantal efficacy is shown in Fig. 3, E to G. Stimulation of axon 1 gave rise to both evoked potentials similar in amplitude to those from axon 2, and a subset (n = 119) of small evoked potentials whose amplitudes were less than any from axon 2. Axon 1 also had a lower quantal content than axon 2 (as indicated by the greater number of failures). In two cells, virtually all of the quantal responses from the weaker axon were as small or smaller than the smallest response from its competitor (Fig. 3, H to J). Among all the multiply innervated cells having an input with small quantal responses, there was a strong tendency (33/37 fibers, 89 percent) for the axon producing the small quantal responses to also have lower quantal content.

In fibers showing small evoked quantal responses, a population of very small spontaneous miniature endplate potentials (MEPPs) was also observed (Fig. 3K). In this experiment, however, the axonal source of the spontaneously released small MEPPs could not be identified (but see below).

The small evoked events observed here were not explained by electrical coupling between neighboring muscle fibers (*13*). The small quantal responses were also not due to release of quanta at a distant site on the muscle fiber (*14*).

With the use of a "floating" electrode (*15*), small quantal responses in multiply innervated muscle fibers were also detected under more physiological recording conditions in the neonatal mouse sternomastoid muscle. Because of the large quantal contents of EPPs in normal physiological saline, quantal efficacy in this case was studied by measuring the amplitude of spontaneously released MEPPs. The spontaneous MEPPs specifically associated with each of the inputs to a multiply innervated muscle fiber were assayed by adjusting the stimulus strength to a suction electrode to stimulate one input repetitively (10 Hz) to cause an

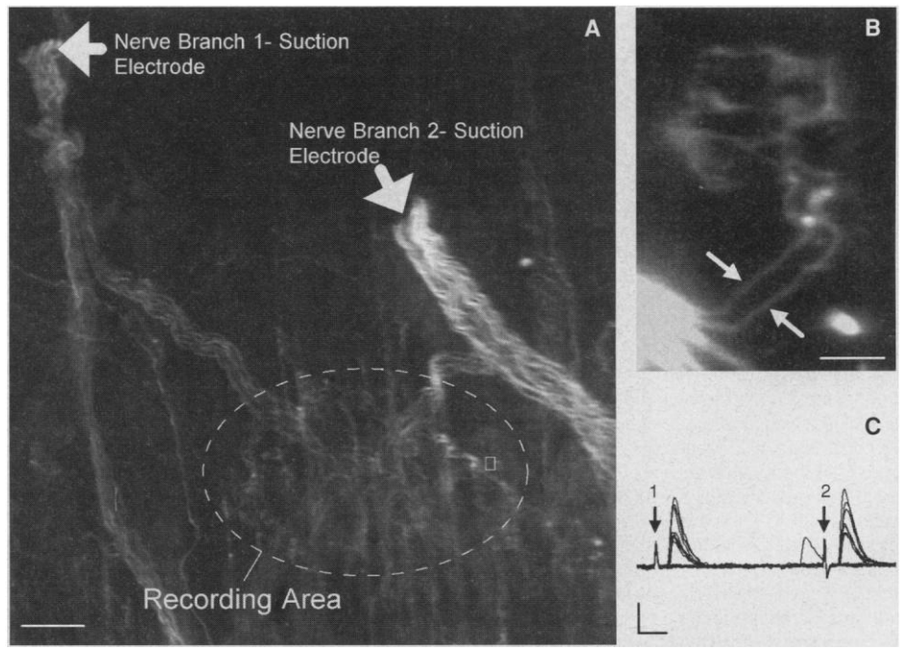

Fig. 1. The neonatal mouse trapezius muscle preparation as used for the physiological studies described. (**A**) A low magnification view of the dissected muscle and fluorescent staining of the nerves with antibodies to neurofilament 200 (*25*) and synaptophysin (*26*) show axons running in two separate nerve branches (large arrows) that converge on a common area of the muscle ("Recording Area"). Normally these branches were left long (1 to 3 mm) to facilitate the placement of suction electrodes on each of the nerve branches (scale bar, 250 μm). (**B**) A multiply innervated junction contacted by two motor axons at high magnification [from the boxed region in (A); scale bar, 10 μm]. (**C**) Synaptic potentials elicited in high magnesium concentration from a multiply innervated muscle fiber. The numbers 1 and 2 indicate the artifacts from stimulation of the two suction electrodes. Each suction electrode was stimulated once during each trace (scale bars, 1 mV, 6.5 msec).

enhancement of spontaneous MEPP frequency from that axon. Repetitive stimulation of a second suction electrode (applied to a different region of the innervating nerve) was used to exclusively activate a second innervating axon.

In a number of instances the spontaneous MEPPs associated with stimulation of each of the two axons were different. Shown in Fig. 4, A to C, for example, is a muscle fiber from a 9-day-old mouse with one strong and one weak input. Repetitive stimulation of the two inputs together gave rise to an increase in the frequency of MEPPs after each evoked potential, which were broadly distributed in amplitude (Fig. 4, D and G). When only the axon that gave rise to the large EPP was repetitively stimulated, large MEPPs (mean = 0.92 ± 0.29 mV) were recorded (Fig. 4, E and H, arrow). Conversely, when the axon giving rise to the smaller EPP was repetitively stimulated, the MEPPs were smaller (0.48 ± 0.20 mV) (Fig. 4, F and I, arrow). As in the case of this example, there was a significant tendency in the population of 148 multiply innervated sternomastoid fibers studied for the axon giving rise to the smaller EPP to elicit spontaneous MEPPs that were on average smaller.

Alterations in synaptic strength in muscles from adult animals. Small MEPPs associated with one input were also observed in 57 transiently multiply innervated muscle fibers in adult muscles undergoing axonal withdrawal that followed reinnervation after the "double nerve crush" paradigm (*16*). As in development, there was a tendency for smaller mean MEPP amplitudes to be associated with weaker inputs to multiply innervated cells; for example, in 77 percent of the cases (44/57) the ratio of the mean MEPP amplitude associated with the weaker (smaller EPP) input divided by the mean MEPP amplitude associated with the stronger (larger EPP) input was <1 ($P < 0.01$, chi-square test). The presence in adult animals of weak inputs with low quantal efficacy argues that the mechanism that removes synapses during development is also operating in maturity.

To determine whether changes in quantal efficacy during synapse elimination could be explained by a reduction in the number of acetylcholine molecules per vesicle, we used repetitive nerve stimulation in the presence of hemicholinium-3 to deplete synaptic vesicles of acetylcholine (*17*); these synaptic responses were then compared with the small potentials observed during development in multiply innervated cells. In singly innervated trapezius muscle fibers from 6- to 9-day-old mice ($n = 5$), nerve stimulation in the presence of hemicholinium-3 and high magnesium decreased the amplitude of evoked quanta to a size that was similar to that observed for the small quantal events in multiply innervated muscle fibers. In all cases, the median rise times of the small events in hemicholinium-3 treated muscles were as fast as or faster ($P < 0.01$, Mann-Whitney U test) than before treatment (*18*). However, the rise times of the small synaptic responses in multiply innervated fibers (in the absence of hemicholinium) were on average slower than the larger quantal responses from the same or the competing axons ($P < 0.01$, Mann-Whitney U test). Thus, the small events observed during synapse elimination are not mimicked by presynaptically reducing the amount of neurotransmitter per vesicle. Data from various preparations, however, have shown that slowing of synaptic responses does occur when the postsynaptic acetylcholine receptor (AChR) density is low (*19*), presumably because of an increase in the time it takes for a neurotransmitter molecule to find an unoccupied receptor (*20*). The reduction in quantal efficacy and slowing of synaptic responses observed in multiply innervated fibers could therefore be related to previous studies showing that, during synapse elimination, the density of AChRs decreases in areas of the postsynaptic membrane that lose nerve terminal staining, and that this postsynaptic change begins before the overlying presynaptic terminal withdraws (*16, 21*).

Given that a low density of AChRs could account for the small quantal responses observed, areas with a low density of receptor staining were identified in neuromuscular junctions in 6- to 8-day-old mice and overlying nerve terminals were studied for signs of synaptic vesicles at these sites. Aggregations of synaptic vesicle proteins were found overlying some areas of decreased receptor density (Fig. 5), consistent with the idea that the small quantal responses observed above are explained by a reduction in postsynaptic sensitivity.

A putative synapse elimination cascade. In conclusion, these studies, in view of previous anatomical work, argue that a cascade of pre- and postsynaptic changes underlie the transition from multiple to single innervation at the neuromuscular junction. In particular, these results suggest that, at birth, most of the neuromuscular junctions are contacted by multiple axons that are similar in both synaptic strength and anatomical area. At some point during the following 2 weeks that varies from one fiber to the next, the synaptic strengths of the inputs at a neuromuscular junction begin to diverge. Perhaps the first change is a reduction in the quantal efficacy at one of the release sites associated with one axon because of a local decrease in AChR density at that site. Shortly thereafter the nerve terminal branch overlying that site is physically withdrawn and, as a consequence, the quantal contents

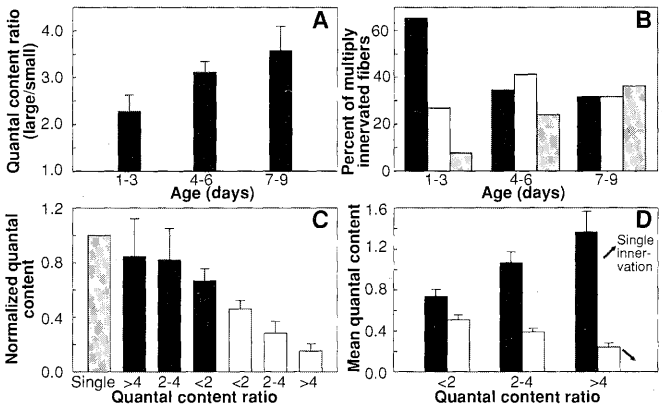

Fig. 2. Evidence for changes in synaptic strength prior to axon withdrawal in early postnatal life. (**A**) The average ratio (large/small, +SEM) of the quantal contents of the two motor axons innervating the same muscle fiber became progressively larger with age. Whereas, at postnatal days 1 to 3, the stronger input had a quantal content that was on average 2.28 ± 0.35 ($n = 27$) times larger than the quantal content of the weaker input, between days 4 to 6 the average ratio was 3.12 ± 0.23 ($n = 75$), and by 7 to 9 days this ratio was 3.58 ± 0.52 ($n = 22$) (all groups significantly different from each other, $P < 0.01$, Mann-Whitney U test). (**B**) The proportion of fibers with inputs of substantially different strengths increases, and the proportion of fibers with inputs of similar strength decreases over the first two postnatal weeks. (**C**) As the strengths of inputs to multiply innervated cells diverge, the larger inputs are preferentially maintained. The average quantal contents (+SEM) of the inputs to multiply innervated fibers are shown as a proportion of the average quantal content of singly innervated fibers (normalized to 1.0) from the same muscles ($n = 42$). The stronger (black bars) and weaker (white bars) inputs for each group are shown. The multiply innervated fibers have been subdivided on the basis of quantal content ratio (that is, <2, 2 to 4, and >4). The stronger inputs to muscle fibers with the largest disparities (>4 times) in quantal content were most alike in strength to the inputs to singly innervated fibers in the same muscles. (**D**) The average absolute quantal contents (+SEM) for the stronger and weaker inputs to multiply innervated fibers plotted as a function of quantal content ratio ($n = 124$). An analysis of individual fibers (not shown) indicated that this average behavior reflected changes in the strengths of both the stronger and the weaker inputs to individual fibers.

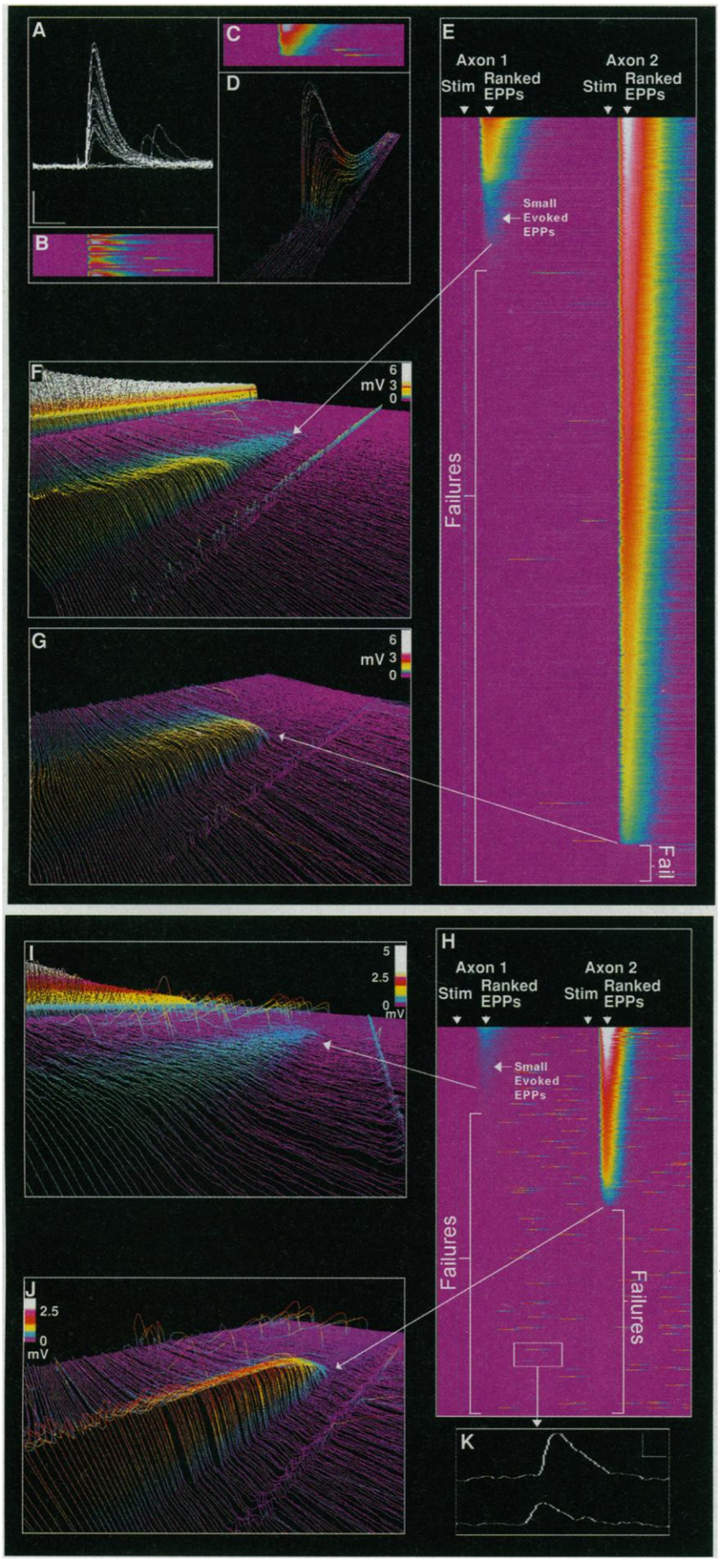

Fig. 3. Small evoked and spontaneous quanta at multiply innervated junctions in the presence of high Mg^{2+}. Each response to nerve stimulation (**A**) was converted to a horizontal line in a digital image (**B**) with the amplitude of the synaptic responses represented by color. (**C**) For each input, the traces were ranked by amplitude such that the largest amplitude EPP is at the top. All physiological traces containing spontaneous MEPPs during or shortly preceding EPPs were removed before analysis. (**D**) The ranked EPPs from (C) represented in height and color. (**E**) The evoked quantal responses from a multiply innervated muscle fiber (6-day-old mouse) innervated by two axons (1 and 2) were arranged independently in order of amplitude. The quantal content of axon 1 is 0.23; axon 2 is 1.20. In addition to the difference in quantal content, the smallest evoked EPPs elicited by stimulation of axon 1 were smaller than the smallest EPPs evoked by stimulation of axon 2. (**F**) The smallest evoked responses from axon 1 [rendered as in (D)] show a gradual decrement in amplitude without an obvious cut-off between the smallest evoked events and failures (in purple). (**G**) The smallest potentials evoked from axon 2 did show a distinct break with the failures. (**H**) A multiply innervated muscle fiber (4-day-old mouse) in which all the evoked quantal responses from one axon were small. The quantal contents of the two inputs differ (axon 1, 0.31; axon 2, 0.91). (**I**) The largest EPPs from axon 1 are comparable in amplitude to the very weakest EPPs from axon 2. The smallest EPPs from axon 1 blend into the failures without an obvious cut-off. (**J**) The smallest potentials evoked from axon 2 show a distinct break between the smallest EPPs and the failures. (**K**) Examples of two categories of spontaneous MEPPs enclosed by the rectangular box in (H). The larger MEPP (1.71 mV) falls within the distribution of single quantal responses from axon 2, whereas the smaller MEPP (0.70 mV) falls within the distribution of EPPs from axon 1 (scale bars = 1 mV, 3.5 ms).

of the two inputs begin to diverge in parallel with a change in their synaptic areas. The same sequence is repeated with increasing rapidity (see below) at other synaptic sites within the junction, causing a further shift in the relative strengths and areas associated with the two inputs.

Such an iterative process would imply that, at a given point in time, the losing axon may possess some synaptic sites that are in the act of being eliminated, while at the same time having other sites that have not yet begun to be affected. The sites undergoing elimination have a low density of AChRs postsynaptically, but still have functional presynaptic terminals. Consequently, release of ACh at these sites would give rise to a subpopulation of quantal responses that have lower efficacy (Fig. 3, E to G). When the losing axon has been whittled down to its last terminal, it too undergoes the elimination process. Thus, the last physiological sign of multiple innervation is the presence of one strong input and one

very weak input that has both low quantal content and uniformly ineffective quanta (Fig. 3, H to J). The very few cases that showed this extreme skewing suggest that it is a very short-lived condition (see below). After this last nerve terminal site is removed, the axon retracts leaving the muscle fiber singly innervated.

Because the relative strengths of two inputs converging on the same muscle fiber changes over time, the ratio of the quantal contents of the inputs is an index of how far the process has progressed on a given fiber. When the inputs are similar in strength, axon withdrawal is unlikely. However, once one input is four times stronger than the other, axon withdrawal of the weaker input would appear imminent. We estimated the length of time that it takes a muscle fiber to become singly innervated once its inputs diverged in strength. The incidence of muscle fibers innervated by two inputs that differed in strength by at least a factor of 2 or a factor of 4 were compared with the number of fibers that lost multiple innervation over the succeeding 24 hours (Fig. 6). This analysis shows that 1 day or less is necessary for fibers to become singly innervated once their inputs differ in quantal content by at least a factor of 4.

The rapid nature of the changes in synaptic strength may be related to the activity dependence of synaptic competition. For example, in a previous study (*22*), AChRs silenced by focal blockade with α-bungarotoxin and the overlying nerve terminals were eliminated only when sufficiently large nearby receptor regions at the same neuromuscular junction were still functional. Those observations argue that the ability of an axon to induce elimination of its competitor is related to its synaptic efficacy. Thus, the skewing observed in our experiments in quantal content and efficacy would be expected to cause further changes in synaptic strength by inducing the elimination of synaptic sites and progressively tipping the competitive balance in favor of one axon over another. In this way, inputs that start out relatively similar in strength could by positive feedback become progressively different over time (as was observed).

It is possible that an analogous sequence of functional and structural changes also accompanies activity-dependent synaptic competition in neuron-to-neuron connections. Interestingly, inputs to neurons undergoing noncompetitive synapse elimination following axotomy (*23*) show changes in quantal content and quantal efficacy analogous to those described above. If similar changes also occur with activity-dependent competition in neurons, then once the strength of the inputs to a neuron were skewed strongly in favor of some subset of inputs by, for example, mechanisms of long-term potentiation or depression (*24*), it is possible that mechanisms similar to those operating at the neuromuscular junction could result in permanent alterations in connections between neurons in the adult brain.

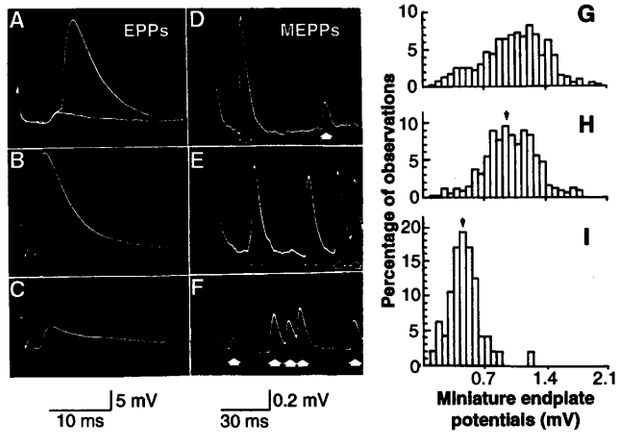

Fig. 4. Small spontaneous MEPPs elicited from the weaker input in normal physiological saline. (**A**) The small EPP, and at higher stimulus strength, the sum of both the small and large EPPs. Stimulation of the larger input alone is shown in (**B**) and the weaker input in (**C**). (**D**) When both inputs were stimulated together repetitively, large (open arrow) and small (filled arrow) MEPPs [at 20 times higher gain than (A)] were seen after each EPP. (**E**) Repetitive stimulation of the large EPP alone was followed mainly by large MEPPs. (**F**) Repetitive stimulation of the small EPP alone was followed mainly by small MEPPs. (**G**) The distribution of MEPP amplitudes (n = 371) after repetitive stimulation of both inputs as in (D) was broad. (**H**) The distribution (n = 308) after stimulation of the large input [as in (E)] was shifted toward higher values, whereas stimulation of the weak input [as in (F)] gave rise to a MEPP distribution (n = 58) with a smaller mean amplitude (**I**).

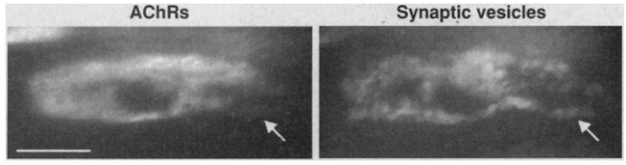

Fig. 5. Synaptic vesicle staining is visible overlying postsynaptic sites where AChR density is decreasing (arrows). In 6- to 8-day-old mouse muscles, postsynaptic AChRs (left) were labeled with rhodamine-conjugated alpha-bungarotoxin, and synaptic vesicles in the presynaptic terminals (right) were labeled with polyclonal antibody G95 to synaptophysin (*26*). Similar results were observed with two other antibodies to synaptic vesicle proteins. Scale bar = 10 μm.

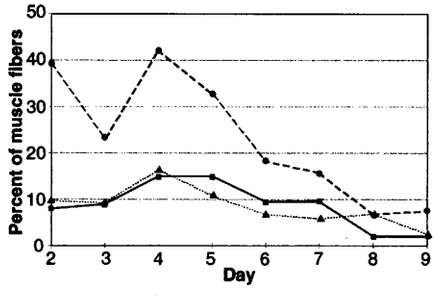

Fig. 6. Synaptic weakening is followed by synapse elimination within several days. Two measures were compared: (i) the percentage of muscle fibers on each day whose inputs differed in strength by some factor [that is, quantal content ratios ≥2 (dashed line) or ≥4 (dotted line)] and (ii) the percentage of fibers that became singly innervated over the succeeding 24 hours (solid line). The number of fibers with two inputs that differed by more than twofold on each day (dashed line) was approximately double the number that became singly innervated by the next day (solid line). Thus, the transition from multiple to single innervation for these fibers must take longer than 1 day. However, the number of fibers whose inputs differed by at least a factor of 4 in strength on each day (dotted line) paralleled the number of fibers that became singly innervated over the next day (solid line).

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