

# Activity-dependent editing of neuromuscular synaptic connections

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**ABSTRACT:** Work over the past four decades has suggested that neural activity edits synaptic connections throughout the developing nervous system. Synaptic editing is shaped in large part by competitive interactions among different inputs innervating the same target cell that profoundly influence synaptic strength and structure. While competition plays out among presynaptic inputs that anterogradely influence their targets, postsynaptic target cells also modulate competition, in part through retrograde interactions that modulate presynaptic neurotransmitter release. One of the most useful synapses for studying how neural activity mediates synaptic editing is the connections between spinal motor neurons and skeletal muscle fibers, called neuromuscular junctions. Here we review current ideas about the role of activity in editing neuromuscular synaptic connections. The mechanisms by which activity mediates synaptic competition at these peripheral synapses are relevant to understanding how neural circuits in the central nervous system are continually altered by experience throughout life. © 2001 Elsevier Science Inc.

**KEY WORDS:** Plasticity, Motor neuron, Spinal cord, Skeletal muscle, Gap junction.

## INTRODUCTION

The exquisite specificity of synaptic connections that is essential for nervous system function arises during development by a series of overlapping phases, including axon outgrowth, pathway selection, target selection and an extended period of synapse formation [36]. These events can occur in the absence of neural activity [90], but work over the last four decades has shown that activity has a profound effect on the subsequent editing of patterns of synaptic connections throughout the central as well as peripheral nervous system [29,45,52]. In regions as functionally diverse as visual cortex [41], cerebellum [24,58], autonomic ganglia [51] and skeletal muscle [9,74], some established, functional synapses are edited out of neural circuits during late embryonic and early postnatal life. This process, often called ‘synapse elimination,’ is shaped by emerging patterns of neural activity that edit nascent patterns of synaptic connectivity so that functional and useful circuits arise as neurons become connected with their targets. Synaptic editing results in some inputs to a target cell becoming gradually weakened and eliminated, while other inputs are strengthened and maintained. Changes in the distribution of synapses between neu-

rons and their targets often culminate in a reduction in the number of inputs innervating target cells, from many inputs to fewer, as in preganglionic inputs to autonomic ganglia neurons [51], as well as from many inputs to a single input, as in motor neuron inputs to skeletal muscle [9,74]. While synaptic editing leads to a frank loss of axonal inputs and synapses, the remaining inputs increase the number and strength of their connections with target cells. Thus synapse formation results in a coarse, but largely correct, pattern of connections between neurons and their targets, while the process of synaptic editing fine tunes these patterns so that they are well-suited to a lifetime of function.

Synaptic editing is mediated by activity-dependent competitive interactions among neurons innervating the same target cell. Competition profoundly influences the strength and structure of synapses from different inputs in a largely Hebbian fashion [38]. Inputs firing coordinately with postsynaptic cells are generally strengthened and structurally reinforced, while inputs that are not are weakened and, in some cases, structurally deleted from neural circuits [38]. While competition plays out among presynaptic inputs that anterogradely influence their targets, postsynaptic target cells also modulate competition, in part through retrograde interactions that modulate presynaptic neurotransmitter release [31]. The activity-dependent anterograde and retrograde interactions that play critical roles in developmental synaptic editing, also play ongoing roles throughout life, and are likely to underlie, at least in part, how changes in experience are translated into changes in brain circuitry.

Seminal studies by Hubel and Wiesel in the developing visual system revealed the role of neural activity in editing patterns of synaptic connections [36,41]. They showed that inputs driven by the two eyes are pitted in competition that is resolved in postnatal life by the segregation of the initially overlapping inputs into alternating bands, called ocular dominance columns, in layer IV of primary visual cortex. The functional loss of binocular inputs to layer IV cortical neurons, as well as the anatomical segregation of inputs from each eye, suggested that some axonal inputs and their functional synapses were eliminated around the time the eyes were first being used. The final outcome of this segregation is exquisitely sensitive to the relative activity patterns of the inputs from each eye during a so-called ‘critical period’ in postnatal life. When one eye was closed so that normal vision was prevented, inputs driven by the closed eye lost virtually all of their synapses with

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layer IV neurons, while inputs driven by the open eye maintained their synapses and expanded their terminal arbors into regions formerly occupied by the closed eye. Closing both eyes had relatively little effect on the distribution of inputs in visual cortex. Thus these experiments were the first to suggest that the relative pattern of activity, rather than the total amount of activity, could determine the long-term viability of synaptic connections. This and related experiments suggest that inputs driven by each eye compete for cortical targets, and that more active terminals from the open eye have a competitive advantage over inactive inputs from the closed eye. Activity manipulations during the critical period produced permanent changes in the synaptic wiring in visual cortex: after the critical period, closing the previously open eye and opening the closed eye, even for years, did not reverse the connectivity in visual cortex. These results were among the first to demonstrate that relatively brief experiences during development could cause permanent changes in neural circuitry.

Relatively little is known about how activity mediates synaptic competition and affects the process of synaptic editing. This is due, in part, to the relative inaccessibility of synapses in the developing brain and to the technical difficulties inherent in studying such dynamic processes. However, at the same time ocular dominance columns are segregating in visual cortex, inputs are being edited on target cells throughout the developing nervous system [36]. In distinction to the visual system, at least some of these areas are less complex and more amenable to studies directed at the site where neural activity has its most profound influence: the synapse. One of the most useful synapses for studying how neural activity mediates synaptic editing is the connections between spinal motor neurons and skeletal muscle fibers, called neuromuscular junctions. Here we review current ideas about the role of activity in editing neuromuscular synaptic connections. The mechanisms by which activity mediates synaptic competition at these peripheral synapses are relevant to understanding how neural circuits in the central nervous system are continually altered by experience, for example during learning and memory formation.

### SYNAPSE EDITING AT DEVELOPING NEUROMUSCULAR JUNCTIONS

During embryonic and early postnatal life, most vertebrate muscle fibers are innervated by several motor neurons, but are typically innervated by a single motor neuron in adulthood [9,43, 52,75,79]. The transition from multiple to single innervation occurs over a several week period after birth. The mature pattern of single innervation of each muscle fiber is essential for the orderly recruitment of motor units during force generation, and is essential for normal motor function [10]. Each axon converges onto the same postsynaptic region, called the endplate, that contains a high concentration of acetylcholine receptors (AChRs). AChRs are clustered by motor nerve terminals via the agrin-MuSK signaling pathway, and this region contains other specializations essential for coupling synaptic transmission to muscle fiber contraction [79].

Differential labeling of the terminal arbors of motor axons converging on the same endplate with lipophilic dyes revealed changes in the deployment of inputs during the transition from multiple to single innervation [1,34]. As multiple innervation of muscle fibers was established, each input had relatively equal presynaptic terminal area and occupied relatively equal areas of postsynaptic AChR-rich membrane (Fig. 1, left). The terminals of each axon converging on the same endplate, which has been termed a "cartel" [52], were extensively intermingled, suggesting that the interactions which determined which cartel was going to be maintained or eliminated, were highly localized within the junction. Physiologic characterization showed that initially each

input has relatively similar synaptic strength as measured by quantal content, the number of neurotransmitter quanta released per stimulus [22], and each appeared to be strong enough to cause muscle fiber contraction when stimulated [9]. The multiple axons that initially innervated the same target cell were equally maintained for a period of several days, and each added terminal branches during this time. These structural and functional observations suggested that multiple innervation is transiently maintained for a short period of time, during which there appears to be a relative balance of power among inputs that will end up competing for sole innervation of each neuromuscular junction.

Around the time of birth, however, the relatively stable deployment of multiple motor neuron inputs changed dramatically. One input maintained its terminals while the others gradually lost their terminals in a protracted, step-wise fashion that lasted several days (Fig. 1, middle). At some junctions, the terminals of competing inputs segregated spatially [34]. This process was not synchronized across junctions; rather, each junction appeared to be at a different stage in the transition from multiple to single innervation. This suggests that the mechanisms driving the loss of an input and its terminals are locally controlled at the level of each junction. The loss of terminals by one axon culminated in its withdrawal from the endplate, leaving the terminals of a single motor axon at each junction (Fig. 1, right).

The use of vital staining and imaging techniques to monitor the same neuromuscular junctions repeatedly in living neonatal animals allowed the shift in the relative balance of power between competing inputs to be studied with fine spatial and temporal resolution. The three cell types that comprise neuromuscular junctions, the motor axons and nerve terminals, muscle fiber postsynaptic specializations such as AChRs and the perisynaptic Schwann cells which cap junctions, can each be stained with fluorescent, non-toxic dyes and imaged simultaneously at neuromuscular junctions [62,71]. By visualizing changes in pre- and postsynaptic elements of junctions innervated by two inputs (the simplest case of multiple innervation), an early event in synaptic competition was observed to involve changes in the density of AChRs within the postsynaptic muscle fiber membrane [2,77]. AChRs were observed to be depleted in small regions of a junction, and this depletion was followed by the loss of overlying nerve terminals. The surviving input maintained a high density of postsynaptic AChRs beneath its terminals. As AChRs were depleted, other postsynaptic specializations, such as rapsyn and related molecules, were also depleted [25]. The remaining input did not expand into the depleted AChR region; rather these sites were permanently deleted from each synapse. Thus it appears that axons do not compete for occupation of the same synaptic territory. This process resulted in a pattern of regions of high AChR density, interspersed with regions devoid of AChRs, that was unique to each endplate. These regions are thus the 'battle scars' of competition that plays out pre- as well as postsynaptically [2].

Physiological evaluation of changes in the strength of competing inputs at dually innervated junctions showed that the quantal content of each input became increasingly disparate. The quantal efficacy of one input was observed to be reduced, and this was interpreted to be due to a loss of AChRs beneath those inputs [22]. While a reduction in the postsynaptic AChR density and presynaptic terminals contribute to the weakening of inputs, neurotransmitter release probability also affects synaptic strength. Thus differences in the probability of neurotransmitter release could contribute to the observed disparity in quantal content among competing inputs. Quantal content and paired pulse facilitation were compared between weak and strong inputs to the same neuromuscular junction. Paired pulse facilitation is a measure of synaptic function that is inversely related to neurotransmitter re-

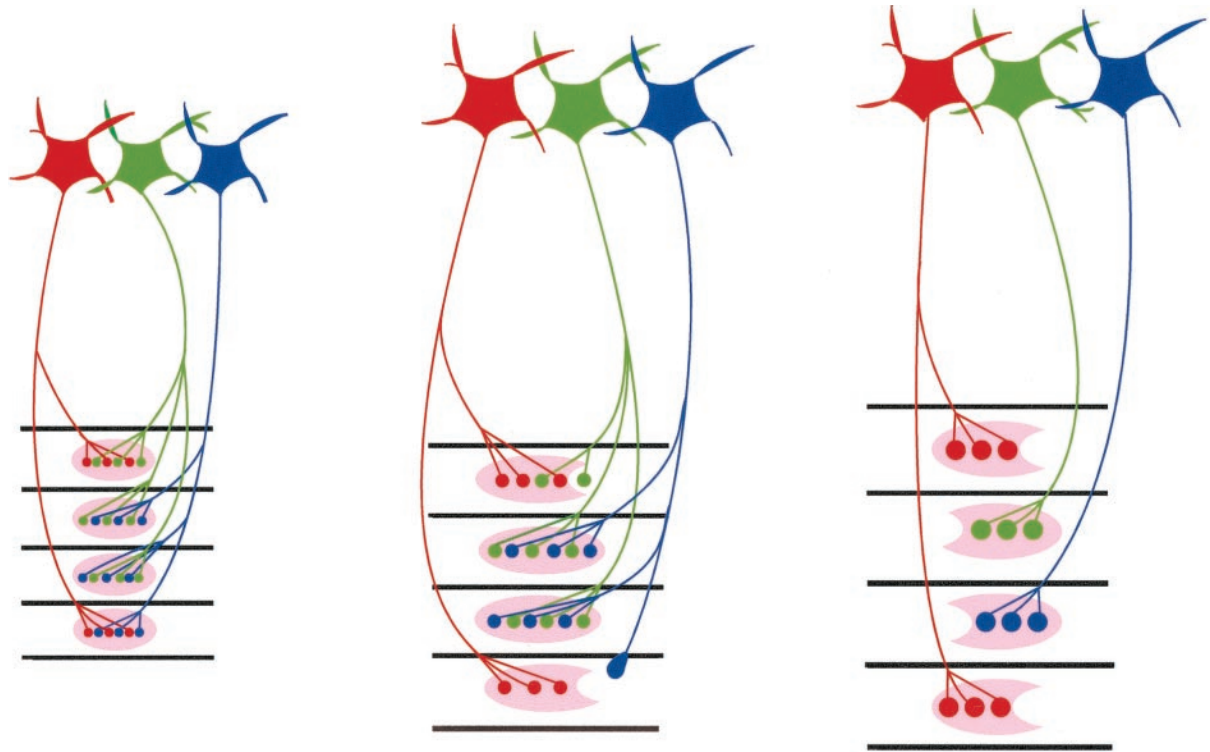


FIG. 1. Transition from multiple to single innervation at neuromuscular synapses. Skeletal muscle fibers undergo a progressive transition from multiple innervation at birth (P0; left) to single innervation typically by 2–3 weeks of age (right). The motor axons that converge on the same muscle fiber have presynaptic terminals that are intermingled over the postsynaptic acetylcholine receptors (AChR) clusters (pink ovals) and occupy relatively equal synaptic area, as well as similar postsynaptic AChR density and synaptic strength. As synaptic competition progresses (middle), some axons lose postsynaptic AChR regions (top fiber) followed by the loss of overlying pre-synaptic terminals (bottom fiber). Loss continues, site by site, until an input loses all of its synaptic area and withdraws from the junction (bottom fiber, blue input). Synapse elimination occurs in the same fashion, but at somewhat different times across all junctions within a muscle, until all fibers are singly innervated (right).

lease probability at neuromuscular and other synapses [44]. Synapses with high release probabilities have a small degree of facilitation, while synapses with low release probability have a high degree of facilitation after paired pulse stimulation. Paired pulse facilitation was greater for the weaker input compared to the stronger input, regardless of their absolute quantal content. This suggests that weaker inputs have a lower probability of neurotransmitter release than stronger inputs to the same junction ([47] and D. Kopp, D. Perkel and R. Balice-Gordon, unpublished observations). These results suggest that differences in presynaptic neurotransmitter release contribute to the increasing disparity in synaptic strength that is a hallmark of the competitive process. A cycle of functional weakening and structural loss continues until all of the sites innervated by weakened inputs are eliminated, and the losing axons permanently withdraw from junctions.

#### ROLE OF NEURAL ACTIVITY IN SYNAPTIC COMPETITION: MEDIATOR OR MODULATOR?

Collectively, the available data on competition at neuromuscular and most other synapses support an activity-dependent positive feedback mechanism in which gradual changes in synaptic strength and synaptic area contribute to an input's long-term viability. These events are summarized in cartoon form in Fig. 2. Inactive inputs that release less neurotransmitter than more active competitors may down-regulate synaptic release machinery.

Postsynaptic AChRs may then become depleted under low release probability sites, resulting in a decreased quantal amplitude [22], followed by the loss of presynaptic terminal regions, continues until losing inputs permanently withdraw from junctions. Active inputs, on the other hand, emerge as winners in the competitive process, by maintaining a high quantal content, in part by maintaining a higher release probability than their competitors. High release probability may, in turn, prevent the depletion of postsynaptic AChRs, preserving synaptic area and strength. After single innervation is established, quantal content increases further until adulthood, probably by the gradual addition of release sites.

The two related questions raised by these structural and functional observations are first, how might the postsynaptic muscle fiber discriminate among initially similar inputs? And second, what mechanisms may induce the strength and the structure of competing inputs to become progressively divergent? These related issues are considered in turn below.

Several lines of evidence suggest that postsynaptic muscle fibers may discriminate among competing "cartels" based on the pattern/timing or overall amount activation of postsynaptic AChR regions. All of the terminals of the same axon, a "cartel," by definition have the same activity, although there may be heterogeneity in neurotransmitter release probability across different terminal boutons. The pattern/timing or the total amount of motor neuron activity impinging on postsynaptic sites, or both, may be

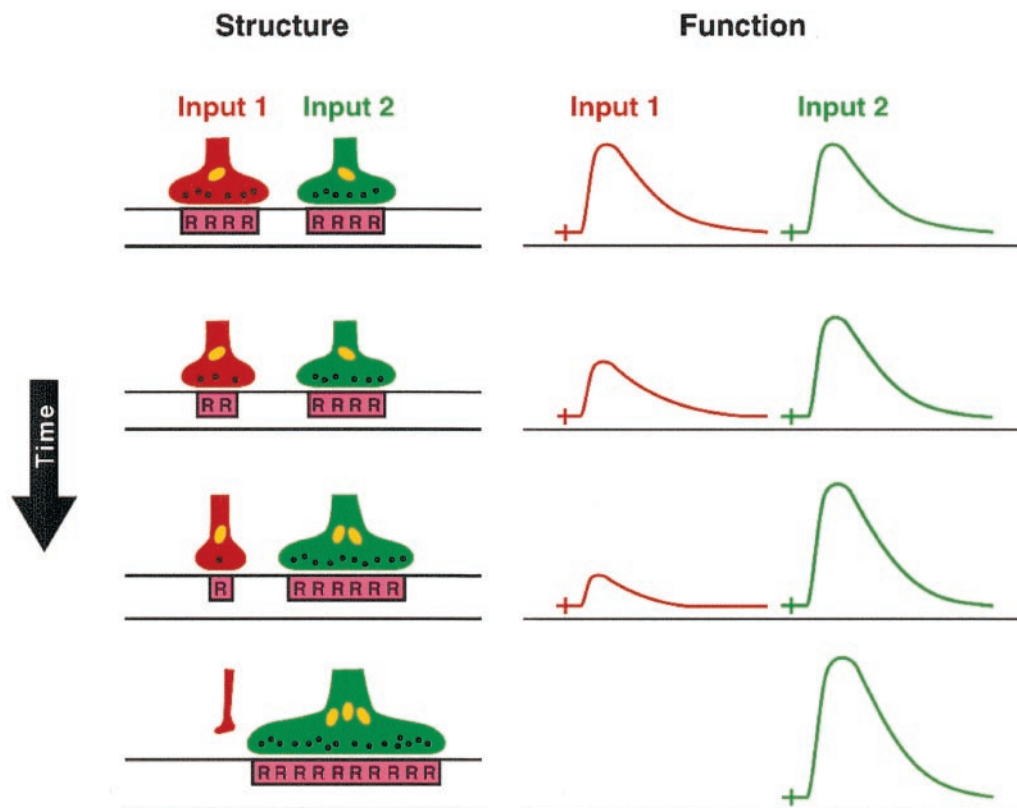


FIG. 2. Cascade of structural and functional changes during synaptic competition at neuromuscular synapses. Early in postnatal life, competing inputs (red and green axons) have relatively equal presynaptic terminal area and postsynaptic acetylcholine receptors (AChR) density (left, top). Each input has similar synaptic strength, as indicated by the relative sizes of their excitatory endplate potentials (epp, top right, red and green waveforms). As competition continues (left, middle), postsynaptic AChR density beneath the one input decreases, followed by loss of the overlying presynaptic terminals until the 'losing' axon withdraws from the muscle fiber (left, bottom, red axon). The 'winning' terminal maintains its pre- and postsynaptic area and survives to singly innervate the junction (left, bottom, green axon). At the same time, epp size becomes increasingly desperate between axons (right, middle).

key determinants of this discrimination. Work by Thompson [86], in which muscles were directly stimulated at 1 and 100 Hz in neonatal rats, showed that the pattern of muscle activation, rather than only the total amount of activation, affected the extent of multiple innervation. However, it remains unclear what patterns and amounts of endogenous motor neuron activity are present during embryonic and neonatal life as synapse elimination takes place.

If all of the motor neurons innervating a muscle fiber were firing relatively synchronously, that muscle fiber may not be able to discriminate among inputs and thus maintain all inputs. If activation of postsynaptic muscle fibers were de-synchronized, as might occur if one motor neuron input was more active than another, the postsynaptic muscle fiber may maintain the more active input and initiate the removal of less active ones. Differential stimulation experiments have suggested that more active inputs can displace less active inputs from muscle fibers [75,78], although qualitatively similar experiments also suggest that inactive motor axons have a competitive advantage over more active axons [16, 76]. Blockade of action potentials in skeletal muscle and nerve, or in nerve alone via a tetrodotoxin (TTX)-impregnated cuff, slows the period of developmental synapse elimination [6,8,87], while increasing action potential activity with stimulation seems to accelerate this process at neuromuscular junctions *in vivo* [68,86] and

*in vitro* [61,66]. Thus, while the overall amount of activity seems to affect the overall rate of synapse elimination, the relative activity among competitors appears to determine the outcome of competition.

The possibility that asynchronous activation of postsynaptic regions might induce synaptic loss was tested by manipulating the activation of small regions of AChRs at singly innervated adult neuromuscular junctions [3]. When AChRs in a small region of a junction were blocked by focal application of  $\alpha$ -bungarotoxin, blocked receptors were observed to disappear over several days, and nerve terminals overlying the blocked AChRs were subsequently withdrawn. The precocious loss of AChRs, prior to nerve terminal loss, mimicked the sequence of events observed during synapse elimination at developing neuromuscular junctions. However, uniform blockade of all of the postsynaptic AChRs did not induce any loss of AChRs or nerve terminals. Thus blocked sites were lost only when active sites were present.

This work suggested several important insights. First, when pre- and postsynaptic activity are not temporally correlated, silent synaptic sites are destabilized. Second, when activation of postsynaptic sites is asynchronous (some AChR regions being activated while others are not), inactive sites are at a disadvantage. Third, since focal postsynaptic blockade was sufficient to induce loss of overlying nerve terminals, there must be retrograde signaling that

leads to stabilization of active presynaptic terminals and destabilization of inactive ones. Finally, this work suggests how less active inputs may be progressively weakened: the temporal mismatch of pre- and postsynaptic activity weakens the less active input, ultimately leading to its loss. That desynchronization of different regions within a junction leads to loss of inactive regions suggests that a similar mechanism may account for developmental elimination, assuming that different inputs are asynchronously active. Conversely, synchronous activation of junctional regions may preclude their loss. While all the sites of one axon would likely be synchronously active, if axons converging upon the same junction were synchronously active, then synapse elimination might be slowed or prevented.

Cangiano and colleagues [13] recently demonstrated that synapse elimination was slowed following synchronous stimulation of inputs to newly formed, ectopic synapses in rat muscle. Busetto et al. [13] showed that multiple innervation was maintained when endogenous motor axon activity was blocked by a TTX-impregnated cuff, and synchronous stimulation of axons was imposed distal to the block, regardless of the overall level of stimulation. Interestingly, if synchronous stimulation was imposed in the absence of nerve block, thus allowing natural action potential activity to occur along with imposed activity, the extent of multiple innervation was the same as in unmanipulated control animals. This result suggests that even a small amount of de-synchronized activity may be sufficient to trigger synaptic competition. These observations suggest that synchronous activation of inputs, or for that matter synchronous *inactivity*, can slow competition, and imply that competition is triggered by asynchronous activity within junctions. Together, the work of Thompson [86], Balice-Gordon and Lichtman [3] and Busetto et al. [13] argues strongly that the relative pattern of activity impinging at neuromuscular junctions, rather than the overall amount of activity, is a key determinant of the outcome of competition.

On the other hand, recent work from Ribchester and colleagues [23] showed that, during the period of synapse elimination that occurs during reinnervation of adult muscle, electrically silent inputs can displace other electrically silent inputs. This suggests that activation of postsynaptic muscle fibers is not required for competitive synapse elimination during reinnervation. However, the interpretation of these experiments is complicated by the effects of paralysis, which affects growth and sprouting as well as withdrawal of inactive axons. Despite this limitation, this work highlights the important idea that activity may be a modulator, and perhaps not a mediator of, competition. Thus it will be of interest to determine whether activity is instructive or permissive for synaptic competition, during development as well as during reinnervation of adult junctions.

#### MOTOR UNIT ACTIVITY DURING THE DEVELOPMENTAL PERIOD OF SYNAPTIC EDITING

In many systems, in particular in the developing visual system, synchronous activity among synapses allows all inputs to be maintained. Spontaneous, temporally correlated activity has been observed in developing neural networks of the retina and spinal cord prior to any sensory experience [30,70]. This implies that neural activity may be relatively synchronous during the time of synapse formation and during the transient period of multiple innervation. However, very little information exists about the temporal aspects of motor neuron firing during the perinatal period when synaptic connections are being eliminated, how these temporal patterns change over time, and how these changes affect the emergence of mature patterns of innervation in muscle.

Distinct motor patterns become apparent during the perinatal

period [69] and several aspects of motor neuron maturation appear to be activity-dependent [42]. A matching of motor neuron and muscle fiber properties also becomes apparent after birth [91]. In newborn rodents, electromyographic (EMG) recordings show that muscle activity is irregular and occurs in protracted bursts [95]. Muscle groups that are reciprocally active in adults are often co-active during early postnatal life, and newborn animals are unable to consistently generate enough force to bear weight. After the first postnatal week, more mature patterns of motor unit activity are observed, though hindlimb function lags behind that of the forelimbs [95]. EMG recordings of single motor units from freely moving adult rats has shown that the natural motor unit firing rates are different across motor unit types, characterized by their force generation, fatigability and fiber type [11,27,40]. However, it is largely unknown when these mature functional classes of motor units and their characteristic firing patterns emerge during development.

To evaluate the possibility that temporally correlated patterns of motor neuron activity are present during the perinatal period when multiple innervation is transiently maintained, and become more uncorrelated during synaptic competition, we have begun to record the endogenous activity patterns of motor neurons during the first days after birth ([72] and K. Personius and R. Balice-Gordon, unpublished observations). The activity of small numbers of motor units (a motor neuron and the muscle fibers it innervates) was recorded in awake neonatal P0–P15 mice using fine tungsten microelectrodes and differential electromyographic techniques [65]. Using fine tungsten microelectrodes, spontaneous motor unit EMG recordings were made in awake, standing, partially restrained animals. Stable recordings could be made for over 1 h, although a given motor unit was rarely active for this entire period. We have focused on hindlimb muscles, because much of the prior work on adult firing patterns has been performed in the soleus and extensor digitorum longus [27,40], and because these muscles have been used extensively for developmental studies of the role of activity in shaping patterns of innervation [86]. Single unit EMG recordings were made from the soleus muscle, because this predominantly slow twitch muscle is tonically active during stance and its motor innervation is not compartmentalized within the muscle. Recording of activity before and after the soleus nerve was cut confirmed that recorded motor unit activity was only from the soleus muscle. A commercially available analysis system (Spike2; Cambridge Electronics, Cambridge, UK) was used to identify 2–5 unique motor unit waveforms within a single channel record. Auto and cross-correlogram analyses were then performed to evaluate motor unit firing patterns and the relative temporal relationships among motor unit firing [80].

At P3–P6, average motor unit firing frequency over the hour-long record was *ca.* 1–2 Hz and increased to 4–5 Hz by P15, when locomotion appears qualitatively similar to that of adults [95]. At P3–5, when greater than 75% of muscle fibers are multiply innervated, around 25% of identified motor units show significant correlation in their firing with another motor unit in the same hour-long recording ( $n = 39$  motor unit pairs). The interval of correlation was broad, with peaks in the cross-correlogram between 1–4 s. When hour-long records were analyzed over shorter intervals of 10–40 s, many periods of highly correlated motor unit firing were observed. At P8–P9, when 50% of muscle fibers are still multiply innervated, and by P14–P15, when synapse elimination is largely complete in the soleus, temporal correlations in motor unit activity were no longer detected (0/24 and 0/53 motor unit pairs, respectively). No relationship was seen between firing frequency and the degree of temporal correlation. Furthermore, correlations were independent of the time course examined in the cross-correlogram (from 5–200 s). Thus temporally correlated

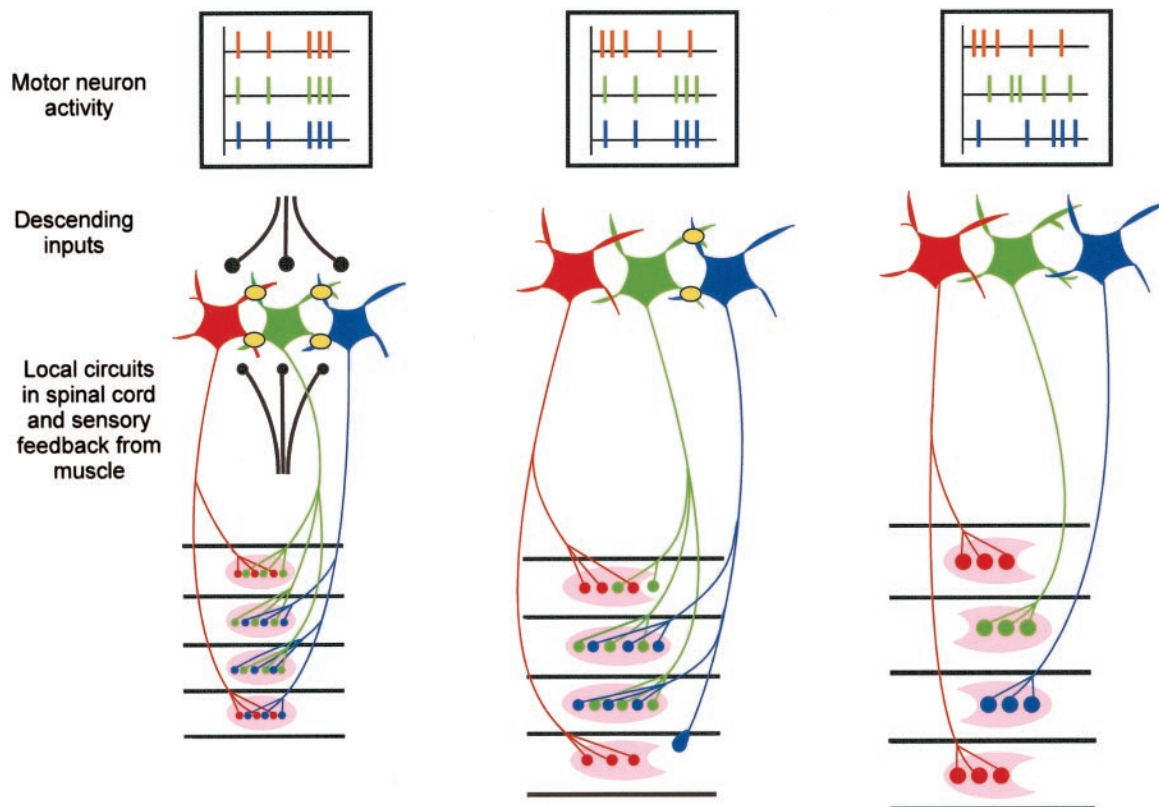


FIG. 3. Roles for temporally uncorrelated neural activity in mediating synaptic editing. During late embryonic and early neonatal development, when each muscle fiber is multiply innervated (left), motor neuron action potential activity may be temporally correlated (left box). Several days after birth, correlated activity among some motor neurons begins to disappear (middle box, red neuron). The loss of correlated activity may trigger synaptic competition (middle). By 2–3 weeks after birth, each muscle fiber is innervated by one motor neuron (right) and motor unit activity is no longer correlated (right box). While the pattern and amount of motor neuron activity may change during postnatal life, in the example illustrated here, the pattern of the red motor neuron is altered, but the total amount of neural input remains constant. The amount and pattern of motor neuron activity may be shaped by the ingrowth of descending cortical and brainstem inputs (thick black lines), by the maturation of local spinal cord circuitry and sensory feedback from muscle (thin black lines), and/or by gap junctional coupling among motor neurons (yellow ovals).

motor unit activity appears to be present relatively early in the process of synapse elimination. We are presently characterizing the extent of correlation in younger animals.

In relating these observations to the patterns of innervation present in adult skeletal muscle, one apparent paradox is that the motor neurons that capture the largest number of muscle fibers (the largest motor units) are those that are the least active: they are recruited last during movements [39]. This has been used to bolster the argument that inactive motor inputs to junctions have a competitive advantage [16,66,75,76]. Barber and Lichtman used modeling to attempt to resolve this apparent paradox, using a few simple assumptions based closely on the cellular observations summarized above [5]. This work suggests that while more active neurons appear to compete more effectively than inactive ones, inactive motor neurons may lose less synaptic territory based on their metabolic advantage. The competitive advantage of a greater amount of activity early in synapse elimination seems to be offset by the relatively greater synaptic efficacy of less active motor neurons later in the process. An alternative explanation may be that there is no recruitment order during development until after synapse elimination, thus the largest motor units do not emerge as the least active until after single innervation is established [12,83]. However, the motor units that start out the largest early in postnatal

life seem to lose the most muscle fibers during the course of competition [4]. Thus it seems plausible that the least active motor units are likely to lose more synaptic targets as a result of synaptic competition than more active motor units.

#### ACTIVITY, GAP JUNCTIONAL COUPLING AND SYNAPSE ELIMINATION IN DEVELOPMENT AND REINNERVATION

The preliminary data summarized above suggest that the relative firing of motor units may be altered during the neonatal period from relatively temporally correlated firing to progressively more uncorrelated firing. There are several possible mechanisms that may underlie this progressive change in motor unit firing, including the intrinsic excitability of motor neurons in a particular pool, local chemical synaptic connections that excite or inhibit motor neurons, descending cortical inputs, the ingrowth of sensory afferents, as well as gap junctional communication via electrical synapses that interconnect motor neurons (Fig. 3). Some of these mechanisms are reviewed elsewhere in this volume [46,48,63]. Here we focus on the possibility that gap junctional coupling among motor neurons may shape their firing and thus influence the activity impinging on neuromuscular junctions.

The presence of gap junctions may bias motor unit firing to be temporally correlated. As gap junctional coupling disappears, uncorrelated activity may emerge. Several labs have characterized the extent of electrical coupling and dye coupling in lumbar spinal motor neurons of neonatal rats [20,33,92]. Intracellular recording shows that neonatal motor neurons are transiently electrically coupled until around P7. Dye coupling is also present until around P7. Injection of Neurobiotin, a low molecular weight compound that passes across most gap junctions, revealed clusters of many labeled motor neurons at P0–P2, but by P7, only single motor neurons were labeled [20].

Motor neurons express five connexins, Cx35, Cx37, Cx40, Cx43, and Cx45 as characterized by reverse-transcription polymerase chain reaction, *in situ* hybridization and immunostaining [20]. This repertoire of connexins is distinct from that expressed by other neurons and glia. All five are expressed during embryonic and neonatal life, while Cx36, Cx37, and Cx43 continue to be expressed through adulthood. The expression of Cx40 sharply decreases after birth. Preliminary work suggests that animals lacking Cx40 undergo synapse elimination several days earlier than wild-type littermates ([19] and Chang and Balice-Gordon, unpublished observations). While preliminary, these results lend support to the hypothesis that the loss of correlated activity among motor units, due to the loss of gap junctional coupling, may trigger synaptic competition, leading to elimination of the least active inputs. It remains to be determined however whether these events are causally related. Thus the mechanisms that shape motor neuron firing patterns, including but not limited to gap junctional coupling (see Fig. 3), may ultimately determine the onset and the outcome of synaptic competition.

Following nerve crush or transection, peripheral axons are capable of regeneration and reinnervation of target cells. Reinnervation of neuromuscular junctions involves the reestablishment of multiple innervation, followed by synaptic elimination that closely resembles neonatal synapse elimination [77]. After nerve transection, adult motor neurons become recoupled by gap junctions [21]. Clusters of several motor neurons are dye-coupled 1 and 4–6 weeks following nerve cut. Electrical coupling was not detected, possibly because the electrotonic distance between dendro-dendritic gap junctions and the somatic recording location is larger in adults than in neonates. The repertoire of connexins expressed by motor neurons following axotomy was observed to be the same as that seen in neonates. The reestablishment of gap junctional coupling may bias the firing of axotomized motor neurons to be temporally correlated. This possibility is being evaluated using electromyographic recordings (Personius and Balice-Gordon, unpublished observations).

#### ACTIVITY SHAPES HETEROSYNAPTIC INTERACTIONS AT DEVELOPING NEUROMUSCULAR SYNAPSES

How might changes in motor neuron activity patterns lead to the progressive disparity in synaptic strength that is a hallmark of synaptic competition? Poo and colleagues have provided several insights from their studies on heterosynaptic interactions at immature synapses in *Xenopus* spinal cord neuron-myoblast cultures [17,18,26,55–57]. Some structural and functional aspects of these synapses in tissue culture are similar to those of neuromuscular junctions *in vivo*. Synaptic contacts made by small neurites have properties of cholinergic synapses, including small, postsynaptic clusters of AChRs, spontaneous release of neurotransmitter [detected as mepps or as miniature endplate currents (mepcs)] and presynaptic stimulation evoked neurotransmitter release with a relatively high quantal content. Moreover, activity dependent interactions among convergent inputs can be demonstrated and eval-

uated in this preparation. In myoblasts innervated by the neurites of two different cells, Poo and colleagues have demonstrated that repetitive stimulation of one neurite (typically 100 pulses at 2 Hz) produces a strong suppression of evoked neurotransmitter release in the second, unstimulated neurite [26,55–57]. Heterosynaptic suppression was observed to persist for up to an hour, which was as long as recordings could be maintained. Heterosynaptic suppression appeared to be presynaptic, in that quantal content was reduced without a change in mepc amplitude or frequency. Similar ‘heterosynaptic’ suppression was observed in myoblasts innervated by one neurite, when a second input was mimicked by iontophoretic pulses of ACh from a pipette, arguing that postsynaptic activation was required as opposed to direct interactions between neurites [17,18,26]. If inactive neurites were stimulated 10–50 ms prior to activation of the second neurite, no suppression was observed [26]. A qualitatively similar phenomenon was reported by Betz et al. in neonatal rat lumbrical muscle, in which differential stimulation of two nerves to the same muscle revealed changes in synaptic efficacy that were interpreted to be due to inhibitory interactions between terminals [7]. Thus the plasticity manifested under these circumstances appears to be largely Hebbian [38,82].

However, synapses in *Xenopus* nerve-muscle cultures are not as differentiated as those *in vivo*; e.g., perisynaptic Schwann cells, which play essential roles in synaptic maintenance and which exchange trophic signals with axons and nerve terminals [88,89], are not present in these cultures. Recent work suggests that glia can potentiate neurotransmitter release from synapses in tissue culture [73]. Neuromuscular junctions *in vitro* are not as robust as those *in vivo*, in that observations can only be made over relatively short times. Thus establishing a link between synaptic weakening and structural loss may not be possible *in vitro*. Thus despite the interesting insights obtained from this tissue culture system, it remains to be demonstrated that similar heterosynaptic interactions occur at intact, neonatal neuromuscular junctions during the time competition and synapse elimination normally occur.

In the developing frog retinotectal system, activity-dependent competition takes place among convergent inputs from the two eyes for innervation of postsynaptic tectal neurons. Poo and colleagues [97] used *in vivo* recordings to show that the timing of pre- and postsynaptic activation affected the strengthening and weakening of synaptic inputs to developing *Xenopus* tectal neurons. Synaptic inputs that were activated repetitively within a 20 ms window before postsynaptic tectal cell firing became potentiated, while subthreshold inputs activated within 20 ms after postsynaptic cell firing became depressed. This work suggested that the temporal order of activation of different inputs to the same target cell could affect synaptic strength. Thus *in vitro* as well as *in vivo*, synaptic strength can be modulated heterosynaptically, by the relative timing of presynaptic firing and postsynaptic activation.

#### LOCAL DESTABILIZATION AND STABILIZATION SIGNALS MODULATING HETEROSYNAPTIC COMPETITION

Heterosynaptic interactions leading to synapse loss are likely to involve signals that destabilize inactive regions as well as signals that protect active ones. These signals may be local, because competing inputs at neuromuscular junctions are extensively intermingled during the transition from multiple to single innervation [1]. The observation that focally blocked sites are lost from junctions, while active sites just a few microns away are not, also argues that the signaling that culminates in the dismantling of inactive synaptic sites must be highly localized within the synapse [3]. On the other hand, action potential generation by muscle fibers may also be important, as subthreshold inputs are those that are

eliminated and tonic, non-twitch muscle fibers that do not generate action potentials have persistent multiple innervation [53].

To account for all of these observations, there are likely to be both stabilization signals and destabilization signals that modulate synaptic competition at developing neuromuscular, and other, synapses [52]. When convergent inputs are synchronously active, both may elicit destabilization signals as well as stabilization signals that essentially cancel each other out. Both inputs may be reinforced by a retrograde messenger(s) that is required to maintain presynaptic quantal content. That these signals act locally and their effectiveness is transient, is supported by observations in *Xenopus* spinal cord neuron-myoblast cultures that inactive synapses were protected from suppression if they were stimulated 10–50 ms before stimulation of the other input or prior to a pulse of ACh [26].

When one input is active but the other is not, destabilization signals produced by the active axon selectively destabilizes AChRs beneath the inactive axon, while the stabilization signal evoked by the active axon protects it. Destabilization of AChRs beneath the inactive axon leads to their structural loss and a subsequent decrease in the effectiveness of the unstimulated input. Retrograde signaling may allow the quantal content in the active input to be maintained.

Although the molecular components of a signaling pathway mediating competitive heterosynaptic interactions are not yet known, one candidate for a localized postsynaptic signal is calcium. In *Xenopus* spinal cord-myoblast cultures, heterosynaptic suppression was induced by releasing caged calcium inside myoblasts, and was abolished by prior loading of myoblasts with the calcium chelator 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA) [17,18]. Interestingly, Cash et al. also showed that release of caged calcium in an uninnervated muscle cell induced suppression of a nearby synapse, suggesting that calcium was involved in the production of a short-range retrograde signal that influenced neurotransmitter release. Taken together, this work suggests that differential activation of a post-synaptic cell produces at least one, and likely many, signals that modulate synaptic strength. Several candidate retrograde messengers have been evaluated for their role in modulating synaptic strength, including nitric oxide, a diffusible gas, which has been shown to mediate synaptic suppression *in vitro* [93]. The *in vivo* role of these pathways is unclear, however. Adenosine triphosphate, arachidonic acid and calcitonin gene related peptide have also been proposed to modulate synaptic transmission at developing junctions, largely by potentiation of spontaneous and evoked neurotransmitter release [32,37,60]. However, the factors that produce the most potent effects on synaptic potentiation, at least in tissue culture, seem to be neurotrophins such as BDNF and NT4/5 that signal through TrkB receptors, NT3 that signals through TrkC receptors, and cytokines such as ciliary neurotrophic factor (CNTF) that signal through other receptor complexes [54,55,59, 84,85,94,96].

The role that neurotrophins and trk receptors play in modulating synaptic structure and function is poorly understood. Recent work from McAllister et al. showed that ligands that signal through trkB and trkC have antagonistic effects in modulating dendritic outgrowth and remodeling in cortical slices *in vitro* [64]. Their results indicate that neurotrophins do not simply maintain neuronal viability, but also play a role in the development of cellular, and thus synaptic, architecture. This is supported by the work of Shatz et al. who demonstrated that ocular dominance column formation was disrupted when either excess neurotrophin [14] or scavengers of BDNF and NT4/5 [15,81] were infused into visual cortex.

The hypothesis that axons compete for a limited supply of trophic factor supplied by their targets has been around for many

years, stemming in part from the well-documented role of trophic factors in mediating neuronal survival. However, for such a mechanism to work, coordinate pre- and postsynaptic activity would have to result in either the local production (unlikely) of a factor only accessible to active terminals, or in local uptake (more likely) of factor by active and not inactive inputs. At neuromuscular junctions *in vivo*, exogenous application of neurotrophins or cytokines [28,49,50] or their overexpression by transgenesis [e.g., of glial derived neurotrophic factor (GDNF)] [67] leads to delays in the time course with which single innervation is established. This may be relatively non-specific, however. For example, excess neurotrophins or cytokines may induce the production of supernumerary axonal branches, and thus indirectly affect the time course of synapse elimination: it would naturally take somewhat longer to remove more convergent innervation produced by more axonal branches.

Despite the accumulation of evidence that suggests that neurotrophins and Trk receptors play a role in neuromuscular synaptic maturation and maintenance, the cellular localization of neurotrophins and trks at neuromuscular junctions, indeed at central nervous system (CNS) synapses, has not been well studied. Gonzalez et al. [35] recently showed that full length TrkB (TrkB.FL), and truncated TrkB lacking the intracellular tyrosine kinase domain required for signaling (TrkB.t1), are localized primarily to the postsynaptic AChR-rich membrane at mouse neuromuscular junctions. *In vivo*, dominant-negative disruption of TrkB signaling, by adenovirus-mediated over-expression of TrkB.t1 in wild-type mouse muscle fibers resulted in the disassembly of AChR rich regions of neonatal as well as adult neuromuscular junctions. This disassembly was qualitatively similar to that observed in mutant mice expressing half as much TrkB.FL as wild type mice. When TrkB-mediated signaling was decreased in myotubes cultured *in vitro* in the absence of Schwann cells and motor neurons, agrin-induced AChR clusters were again disassembled. This suggested that TrkB-mediated signaling in the muscle fiber membrane affects the maintenance of AChR clusters, even in the absence of other neuromuscular junction components. Because down-regulation of endogenous TrkB.FL regions causes AChR cluster disassembly, it will be of interest to determine whether over-expression of TrkB.FL in muscle fibers has the opposite effect (i.e., inducing enlarged AChR clusters). It will also be of interest to determine whether over-expression of TrkB.FL prevents or attenuates the normal loss of AChR regions that is a hallmark of synaptic competition at developing and reinnervated adult neuromuscular junctions. These are two aspects of ongoing work aimed at elucidating the cascade of molecular events linking motor neuron activity to changes in synaptic strength and structure.

## SUMMARY

A common feature of developmental plasticity, in the central as well as peripheral nervous system, is that temporally correlated pre- and postsynaptic activity appear to produce strengthening of synaptic connections, while uncorrelated activity produces weakening. At neuromuscular junctions, a decrease in synaptic strength involves presynaptic changes in neurotransmitter release and postsynaptic changes in neurotransmitter receptor density that culminate in the withdrawal of weakened inputs from junctions. Similar mechanisms may account for structural and functional plasticity commonly observed at neuron-neuron synapses in the developing and mature brain. If changes in synaptic strength within the CNS also result in the permanent loss of ineffective inputs, this would provide a mechanism by which changes in synaptic function would become permanently etched in neural circuitry.



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