

Synaptic plasticity at developing neuromuscular junctions: role of the timing of spike activity in the competing inputs

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ABSTRACT

Temporal spike correlation in pre- and post-synaptic cells strengthens or weakens synapses in development or in cellular models of learning (long-term potentiation and depression), two well-known paradigms being Hebb's postulate and spike-timing-dependent plasticity. A favorable model to investigate synaptic modification is the input elimination that occurs at developing neuromuscular junctions as a result of competition between the motor nerve terminals. Activity influences this process, but its precise role remains controversial. Here we present a series of studies in which we address the role of spike timing in the competing inputs: we provide evidence that synchronous activity blunts competition among motor nerve terminals while an asynchronous one strongly activates competition leading to synapse elimination.

Key words

Synapse elimination • Synapse formation • Neuromuscular junction • Activity-dependent plasticity • In vivo electrical stimulation • Chronic conduction block

Introduction

The development of the nervous system is based on genetic as well as epigenetic mechanisms: among the latter a prominent role is played both by chemical factors (neurotrophins being an important class) and by the electrical impulse activity. The result of these developmental interactions is the complex set of synaptic connections that characterize the adult nervous system. One remarkable aspect of embryonic development is the initial formation of profuse synaptic connections, followed during late

embryonic and early post-natal life by withdrawal of a surprisingly high number of albeit fully functional synaptic inputs. Meanwhile the remaining inputs enlarge and become stronger. It is of great advantage for investigative reasons that these processes, collectively known as "synaptic elimination", occur not only in the CNS but also in the much simpler and accessible structures of the PNS and in particular at the neuromuscular junction (NMJ) (for review: Katz and Shatz, 1996; Buffelli et al., 2004; Tapia and Lichtman, 2008). Here synaptic elimination determines, in all muscles, a shift from the poly-neuronal

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innervation of late embryonic and early postnatal NMJs, to the well-known mono-neuronal innervation of the adult: each muscle fibre is contacted by only one of the many intramuscular collaterals of a given motoneurone (Fig. 1A). For practical purposes it is useful to specify that in rodents, the choice animals for these studies, the adult condition is reached about two weeks postnatal (Redfern, 1970; Bennett and Pettigrew, 1974; Brown et al., 1976).

Although synaptic elimination was discovered at the NMJ (Redfern, 1970), and its description at the fine morphological level is by far the most advanced than at any other synapse (Balice-Gordon and Lichtman, 1993; Kasthuri and Lichtman, 2003; Walsh and Lichtman, 2003), knowledge of causal mechanisms and signals involved have not progressed to the same extent (see Discussion). Thus, it is from a much more complex CNS model, postnatal development of visual cortical connections, that meaningful physiological mechanisms have been proposed: these stem from Hubel and Wiesel experiments of visual deprivation and eye's misalignment in kittens (Wiesel and Hubel, 1963; Hubel and Wiesel, 1965), and indicate a model of activity-dependent competition between synaptic inputs from the two eyes on common target cortical neurons: synchronous activity blunts competition while an asynchronous one promotes it. Because of the advantage and interest in investigating basic mechanisms of competition at the NMJ, we got inspiration from these experiments for devising a series of investigations that we implemented over several years and review here. Some of the experiments use electrical stimulation to induce synchronous activity: these were also encouraged by the marked effects of electrical stimulation on the distribution of synaptic inputs and postsynaptic AChR clusters that form under the influence of a foreign nerve in soleus muscle (Lømo and Slater, 1980; Lømo et al., 1988). Other experiments are on spontaneous activity of motor units in newborn rats.

Methods

Animals and surgery

The experiments were carried out on two groups: adult and newborn rats. In adults, we observed the effect of synchronous spike activity, evoked by electrical nerve stimulation in the axonal inputs to mus-

cle fibres, during the period of synaptic elimination that accompanies muscle reinnervation. In newborn rats, we recorded through electromyography (EMG) the changes in the temporal pattern of spontaneous spike activity of motor units during perinatal development. All experiments were carried out on male Wistar rats and were authorized by the Istituto Superiore di Sanita' and the Ministry of Health of Italy. All surgery was performed under general anesthesia, either ether or equithesin, as described in detail in the original papers (Busetto et al., 2000; Buffelli et al., 2002; Favero et al., 2010).

Chronic electrical stimulation

We performed chronic stimulation of the axons that reinnervate soleus and extensor digitorum longus (EDL) muscles following section or crush of their original innervation in two different paradigms, described in detail below. The general purpose is to establish a synchronous spike activity in the regenerating and reinnervating axons during the period of polyneuronal innervation and synapse elimination that typically occur in adult muscle after denervation, as a recapitulation of development. To obtain pure synchrony, in all rats of both paradigms the sciatic nerve is blocked chronically with TTX perfusion through cuffs and mini-osmotic pumps implanted *in vivo* (Pasino et al., 1996; Busetto et al., 2000; Favero et al., 2010), to prevent that the spontaneous firing of adult motoneurons, asynchronous in nature (Buffelli et al., 2002. For review: Burke, 1994; Rothwell, 1994), reaches the muscle.

In a first paradigm, the soleus muscle was denervated by cutting the original nerve which was also prevented from reinnervating the muscle: reinnervation occurred instead by a foreign nerve (fibular) previously transplanted on a non-synaptic region located midway between original endplate zone and proximal tendon (Busetto et al., 2000). In a second paradigm, the muscle (soleus and EDL, but in different rats) was also denervated, but reinnervation by the original nerve could take place due to its crush placed close to the muscle (Favero et al., 2010). Electrical stimulation is by means of series of supra-maximal rectangular pulses, each evoking an action potential in all motor axons of the muscle nerve, organized in trains which reproduce as closely as possible the pattern and amount of daily activity (see Results and for details: Busetto et al., 2000; Favero

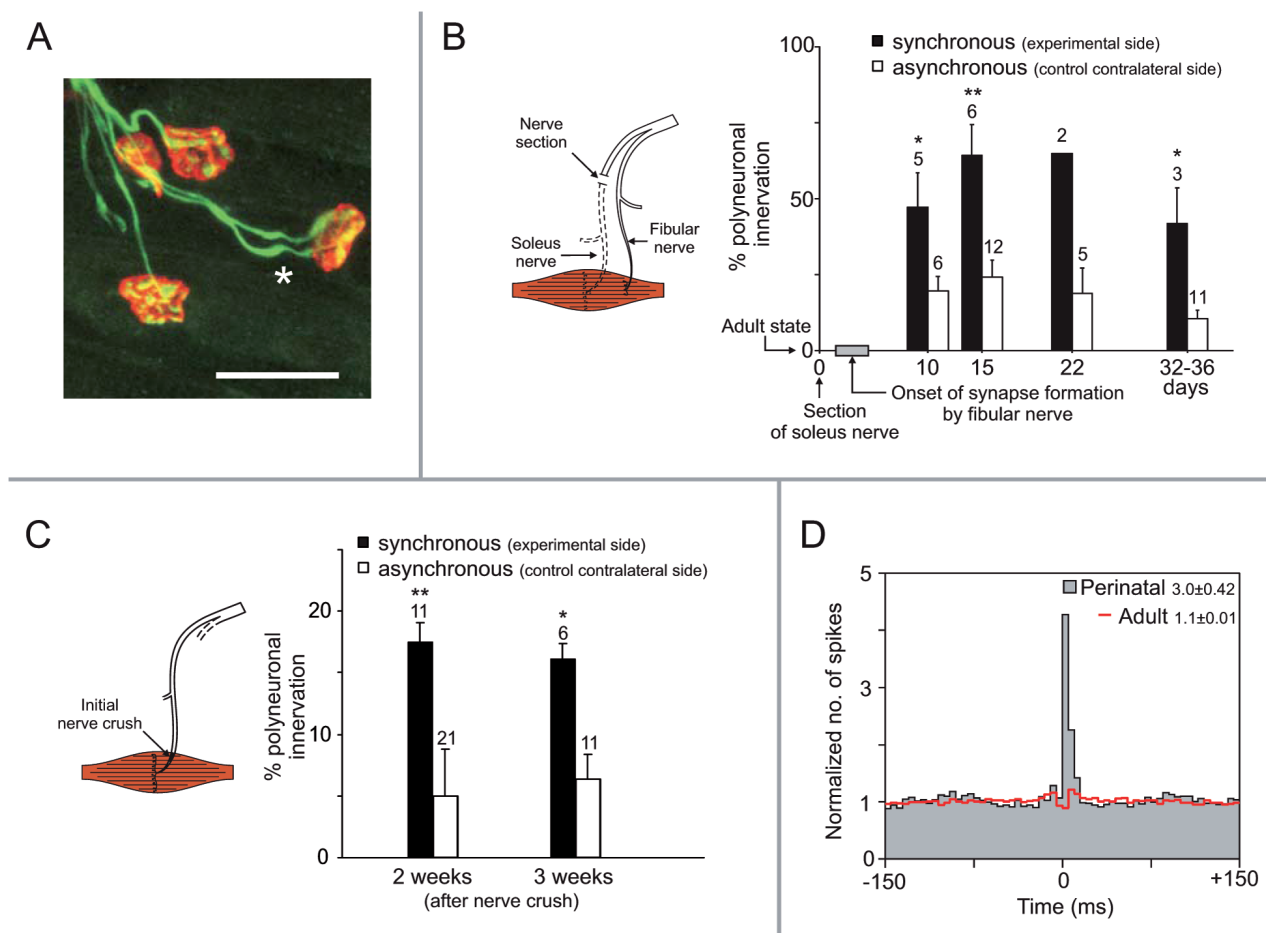


Fig. 1. - Activity-dependence of synaptic elimination: importance of the timing of spike activity in the competing inputs, in rodents. **A**: developing soleus muscle at P14 showing mono-innervated fibres and a poly-innervated one (*), at fluorescence confocal microscopy. Axons, green; terminals, yellow; AChR aggregates, red. Bar: 25 μ m. **B**: time course of polyneuronal innervation in experimental and control soleus muscles, in an adult model of synaptic elimination at ectopic fibular NMJs, following section of the soleus nerve. Number of muscles above columns. * $P < 0.05$, ** $P < 0.005$. **C**: similar to B, but with reinnervation of the original endplates following crush of the EDL and soleus nerves close to the muscle entry point. **D**: average cross-correlograms of couples of TA and soleus EMG motor units, recorded between E21 and P31 in normal rats. Abscissa: intervals between unit signals within each couple (see text). Ordinate: number of spikes for each interval class, normalized to the baseline number equaled to 1. Bin width: 5 ms. Gray area: perinatal couples (30; E21-P5). Continuous red line: adult couples (47; P13-31). Correlation index k' in upper right corner.

et al., 2010) prevailing physiologically (Hennig and Lømo, 1985).

The stimulation is by means of electrical wires (terminating in close proximity to the appropriate nerves) that come from a stimulator placed outside the animal through electrical swivel and tether (Busetto et al., 2000; Favero et al., 2010).

EMG recordings from developing muscles at perinatal stage

Depending on age, extracellular short micropipette tips filled with 4 M NaCl, floating, or thin 25 μ m

platinum wires, were used for EMG recordings of motor unit potentials (Buffelli et al., 2002). Tibialis anterior (TA) and soleus muscles were exposed through short skin incisions under ether anesthesia and the electrodes placed in the muscles: after recovery from anesthesia, recordings started under partial contention of the animal that permitted spontaneous flexion and extension hind-limb movements (Buffelli et al., 2002). Recording sessions of 30-60 min terminated with animal death by excess ether. Embryos extracted from the uterus at E21 and newborn animals from P0 to P31, were used for

the EMG recordings. Rats \geq P13 are labeled here as adults, because synaptic elimination is essentially complete by this age. Techniques of EMG unit analysis are described in detail elsewhere (Buffelli et al., 2002).

Morphology and in vitro electrophysiology

Techniques of histology and confocal fluorescence microscopy have been described in our previous publications (Busetto et al., 2000; Buffelli et al., 2002; Favero et al., 2010), to which we defer the reader: they permitted the visualization of the transition from polyneuronal to mononeuronal innervation and also some quantitative assessments of these processes. However, most of this quantitation was based on *in vitro* electrophysiology, which was performed at the end of the chronic period of nerve electrical stimulation. Graded single shock stimulation of the muscle nerve was performed *in vitro* while recording the evoked endplate potential (EPP) from the muscle fibres with intracellular micropipettes, while the firing of the muscle action potential was prevented by μ -conotoxin and/or critical doses of curare: polyneuronal innervation was signaled by multiple EPP steps (Busetto et al., 2000; Favero et al., 2010).

Statistics

Results are expressed as mean \pm standard error of the mean (SEM). Significant differences are based on Student's two-tailed *t*-test. P-values < 0.05 are considered significant.

Results

General features of experimental conditions

The aim of the present studies is to investigate the role of the timing of spike activity in the motor terminals that compete during the developmental period of synapse elimination, to remain the only input of any given muscle fibre. Ideally, this would amount to electrical stimulation *in vivo* day and night of the nerves giving rise to polyneuronal innervation during the first 10 days or so of postnatal life, a period where the bulk of elimination occurs. This is clearly in itself a formidable task, made even worse by the necessity of keeping the axonal conduction

continuously blocked central to the stimulation site, to eliminate asynchronous activity from reaching the NMJs (sciatic nerve TTX perfusion, Methods). However, the sequence polyneuronal innervation-synapse elimination is recapitulated in the adult condition, when muscles are reinnervated following damage of their innervation. Because of animal size, this opened the way to our experiments, which we applied in two reinnervation paradigms: ectopic and original synaptic sites.

Activity-dependent synaptic elimination at ectopic, distributed NMJs

A few days following section of the original nerve, transplanted fibular axons start to form synapses on newly formed acetylcholine receptor aggregates (AChRs) distributed over some distance on given muscle fibres, some inputs converging on the same aggregate (Lomo, 2003, for review). Our chronic stimulation induces a synchronous spike activity in the competing axons during the entire chronic *in vivo* period that lasts for a variable time, up to 36 days following soleus nerve section (Fig. 1B). After this time period, soleus muscle and fibular nerve are isolated *in vitro* and the percentage of polyneuronal innervation of muscle fibres is determined electro-physiologically. Comparison is made with contralateral control muscles that are characterized by similar reinnervation by the fibular axons, without however chronic sciatic nerve conduction and distal stimulation of the axons: this way, reinnervation occurs with inputs that retain the physiological pattern of spike activity, which in the adult is asynchronous in nature (Buffelli et al., 2002. For review: Burke, 1994; Rothwell, 1994). The stimulation parameters used on the experimental side are chosen to reproduce the type of activity prevailing in the control side. Since the fibular axons normally innervate fast muscles (peroneus brevis and longus), best estimates for activity level of their motoneurons can be obtained from Hennig and Lomo (1985) who recorded *in vivo* motor unit firing of the fast EDL. Accordingly, we used total number of stimuli day⁻¹ ranging from $\sim 11,000$ to $\sim 86,000$ and frequencies of 80 Hz (Busetto et al., 2000, for details).

On the control side, we started recording 10 days after soleus nerve section and detected several polyneuronal innervation of muscle fibres in the region of the fibular nerve implant; their value reached a maximum

of ~1/4 of the fibres around day 15 and then slowly declined through the process of synaptic elimination. At the latest time explored, some polyneuronally innervated fibres were still detectable (Fig. 1B). This essentially reproduces the developmental events of perinatal times. Only to be noticed: 1) the lower level of maximum poly-innervation reached in this adult model, because in development all fibres are initially poly-innervated, 2) the fact that after > 1 month of reinnervation there are still poly-innervated fibres, but we do know from previous work that this is a characteristic of this distributed model of adult reinnervation (Lomo, 2003), 3) the distributed nature of the inputs, which will be dealt with in the next section.

The important result of this series of experiments is that in the experimental muscles, where the activity is synchronous, the percentage of poly-neuronally innervated fibres is much higher than in the control ones, maintaining a 3-fold level for a prolonged time period (15 and 22 days, Fig. 1B).

Activity-dependent synaptic elimination at NMJs where inputs converge on a single synaptic site

The previous model has the advantage that the synapses on which we test the effects of synchronous activity, are newly formed in both pre- and post-synaptic components, like in development. However, the poly-innervating inputs are distributed over a relatively long stretch of each muscle fibre, instead of being concentrated on a single small AChR endplate area. Thus we repeated the experiments in a model where reinnervation takes place after crush of the original nerve: the regenerating axons follow the degenerating paths and regain the original endplate (Rich and Lichtman, 1989; Ribchester, 1993). We used soleus and EDL muscles. The stimulation protocols in the experimental preparations were such to reproduce the amount and firing frequencies of the activity of the motoneurons that reinnervate the control ones: like in the ectopic paradigm, the latter are in fact reinnervated by axons undergoing natural spike activity. Thus, stimulation frequencies are appropriate for fast EDL (80 Hz) and slow soleus (20 Hz), while amounts ($86,400 \text{ day}^{-1}$) are nearly maximal for EDL and substantial for soleus (Hennig and Lømo, 1985).

The results in EDL and soleus muscles are similar and their data pooled together in Fig. 1C. Both at 2

and 3 weeks post-crush (reinnervation starts early: day 3-4), the stimulated preparations exhibit a definitely higher polyneuronally innervation percentage than control preparations. The EPPs in poly-innervated fibres have an identical time course, confirming that the competing inputs converge on the same endplate, like in developing NMJs (not shown).

Firing patterns of motor units in newborn rodents and the process of synaptic elimination

An important question arising from the striking effects of the imposed synchronous activity on synaptic competition, is what is the pattern of activity of motoneurons during the period of developmental, physiological competition. To this end, we recorded from awake late embryos (E21) and early postnatal rats (P0-P13) single unit EMG activities. We also compared them to “adult” rats (P13-P31), to investigate if changes in activity pattern take place, that could shed light on the activity-dependence of synaptic elimination. Muscles investigated are tibialis anterior (TA) and soleus, as examples of foot flexors and extensors, respectively.

We recorded EMG motor unit signals, which indicate the firing of single motoneurons, simultaneously from couples of motor units. The cross-correlation inside these pairs is determined by measuring, over a period of ~2 min, the intervals between each time one unit fires taken as reference (time 0) and all those of the other unit, preceding or following within a 150 ms window. A higher probability of a given interval/s appears as a peak over the baseline, that is the chance level, a peak near 0 indicating a trend towards synchrony. To measure the strength of the correlation we used the correlation index k' (Ellaway and Murthy, 1985), 1 indicating no correlation.

We found that in adult rats no correlation is detectable, as k' is on average 1.1 ± 0.01 in 47 EMG unit pairs from 12 muscles (P13-P31) (Fig. 1D, red line), confirming the well known asynchronous firing characteristic of the physiology of normal muscles (Burke, 1994; Rothwell, 1994). What is surprising instead, is that a correlation clearly exists at early developmental times, k' showing maximum values close to 8 just after birth. Averaging out all correlograms of 30 pairs taken from 17 muscles at early times (soleus E21-P5, TA E21-P2), a striking peak appears near time 0, the average k' being 3.0

± 0.42 (Fig. 1D, gray area). The width of the peak approaches 25 ms, indicating that at perinatal times different motoneurons of the same muscle tend to fire in tight synchrony. Another finding supports this conclusion. When the movements intensify, it becomes impossible to demonstrate the firing correlation through single unit analysis, because too many units are recruited. However, in newborn animals a characteristic grouping of spikes exists, clusters regularly spaced by pauses, an expected feature of motoneuron synchronization. Consistently, we observed no grouping in the adults, where motoneurons are desynchronized. In agreement with our findings is that a reduced gap junctional coupling was reported to cause desynchronization and elimination (Personius et al., 2007).

Our further findings are that the “desynchronization” of motor units occurs a few days earlier in TA than in soleus muscles ($\sim P3$ in TA and $> P5$ in soleus), and that synapse elimination quantitatively determined with confocal microscopy is also time-dependent, occurring earlier in TA muscles (Buffelli et al., 2002).

Discussion

The experimental approaches described here, from different viewpoints illustrate the activity-dependence of the process of synaptic competition and elimination during formation of neuromuscular connections. Their most significant contribution to the field rests on focusing on the timing of action potential activity in the competing motor inputs, an important physiological aspect not previously specifically addressed (see discussion of Buffelli et al., 2002, for further information on this point). In fact a number of previous reports investigated the effects of overall neuromuscular activity changes (Benoit and Changeux, 1975; O'Brien et al., 1978; Thompson et al., 1979; Brown et al., 1981; Thompson, 1983; Nelson et al., 1993), but their results have more to do with early development, that is establishment of muscle innervation, which characterizes the embryonic life or adult repair of connections through neurotrophin regulation (Pittman and Oppenheim, 1979; Thompson, 1983; Brown, 1984; Levi-Montalcini, 1987; Jansen and Fladby, 1990; Dahm and Landmesser, 1991; Gautam et al.,

1996; Snider and Lichtman, 1996; Nguyen et al., 1998; Busetto et al., 2000). Other studies have, more appropriately, dealt with the differential activity of competing inputs, namely active vs. inactive ones, with contradictory findings about who are the winners (Ribchester and Taxt, 1983; Ridge and Betz, 1984; Callaway et al., 1987; Balice-Gordon and Lichtman, 1994). Finally the controversy has been resolved in favor of the active inputs (Buffelli et al., 2003): it still remains to be investigated what happens when all competing inputs are active, which is the physiological feature prevailing during developmental synaptic elimination. This is why the timing of spike activity becomes a crucial issue, which has been addressed by us both with electrical stimulation experiments and with recordings of spontaneous spike activity of motoneurons during development. Although the stimulation experiments were, by necessity, performed in adult animals in which reinnervation recapitulates development, nonetheless their indication is clear: synchronous activity markedly weakens competition and prolongs the duration of polyneuronal innervation, whereas an asynchronous one stimulates competition, leading to synaptic elimination. It must be emphasized that the amounts of daily spike activity are comparable in the two paradigms, yet their effects on elimination are dramatically different, as seen when experimental are compared to control muscles in both the ectopic and the original synapse reinnervation models. Also important in this connection are recent experiments of our lab that directly demonstrate this point: identical number of stimuli day⁻¹ applied to competing nerves (in a peculiar rat strain were the soleus muscle is reinnervated by two nerves, soleus and aberrant), powerfully activate competition if applied in an asynchronous manner, although suppressing it if applied synchronously (Favero et al., 2007).

The other line of investigation, recording of spontaneous motoneuronal firing at perinatal times, completes our understanding of the role played by spike timing in synaptic elimination. The demonstration of an early tight synchronous firing is a novel one, and its role may be interpreted as a means to prevent too early competition between incoming inputs, thus allowing all motoneurons to have access to a sufficient share of the muscular territory. The quick desynchronization that follows, on the other hand, well explains the switch-on of the competition and of the

elimination of redundant inputs. The comparison of the time of onset of de-synchronization of TA motor units with that of soleus ones, nicely fits with this scheme, because 1) it occurs in both muscles slightly earlier than the bulk of synaptic elimination, and 2) it does it appropriately earlier in TA than in soleus. Other signals have been implicated in synaptic competition and elimination, such as activity-independent factors (Costanzo et al., 2000) or synaptic strength (Buffelli et al., 2003). They will have to be integrated, however, with our demonstrated role of the timing of spike activity.

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