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THE TIMING OF IMPULSE ACTIVITY SHAPES THE PROCESS OF SYNAPTIC COMPETITION AT THE NEUROMUSCULAR JUNCTION

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Abstract—The development of neuromuscular junctions exhibits profound remodeling that brings from an immature state characterized by multiple motoneuronal inputs per muscle fiber, to a mature mononeuronal innervation. This striking elimination process occurs both perinatally and during adult reinnervation, and is also widely present in the developing CNS. The accelerating influence of the amount of impulse activity on this process, has been shown by various studies, but a more subtle role of the time correlation of action potential firing in the competing inputs, has also been suggested. Here we explore the latter influence using a rat adult model of neuromuscular junction formation, that is reinnervation following a motor nerve crush. This shares all important features with perinatal development, especially the strict juxtaposition of the competing inputs. In fact the regenerating axons converge on a single cluster of postsynaptic receptors, that is the original endplate of each muscle fiber. This focus on the spatial aspect of competition between nerve endings was missing in our previous experiments employing a similar paradigm. We impose a chronic synchronous firing to the competing terminals, by *in vivo* electrical stimulation of their axons distal to a sciatic nerve conduction block. Control preparations, with similar post-crush reinnervation, are left with their natural impulse activity unperturbed. We find that the experimental muscles display a prolonged duration of polyneuronal innervation with respect to controls, indicating that hebbian mechanisms participate in the synapse elimination process. Another aspect dealt with in our study is the genuine nature of the polyneuronal innervation occurring during adult muscle reinnervation, because it is supported by both confocal microscopy and by appropriate electrophysiological tests that exclude electrical coupling of myofibers by gap junctions. © 2010 IBRO. Published by Elsevier Ltd. All rights reserved.

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Neural activity modulates synaptic connectivity in both immature and mature nervous system. This plasticity spans from slow developmental changes to fast modifications in synaptic function and structure underlying memory formation, opposite processes of construction and elimination making a contribution (Bliss and Collingridge, 1993; Katz and Shatz, 1996; Lichtman and Colman, 2000; Bi and Poo, 2001). The neuromuscular junction (NMJ) is among the investigated preparations, for greater simplicity and the occurrence of synapse competition and elimination, whereby the poly-neuronally innervated embryonic muscle fibers attain the adult mono-neuronal innervation during early postnatal life (Redfern, 1970; Bennett and Pettigrew, 1974; Brown et al., 1976; Betz et al., 1979; Kasthuri and Lichtman, 2003. For review: Sanes and Lichtman, 1999; Buffelli et al., 2004; Tapia and Lichtman, 2008). This extreme evolution up to a final mono-neuronal innervation also occurs in some CNS synapses (Mariani and Changeux, 1981).

Early studies suggested that synapse elimination is activated by the increased level of muscle activity accompanying innervation, because it is delayed by muscle paralysis (Benoit and Changeux, 1975; Thompson et al., 1979; Brown et al., 1981) and speeded up by a stimulation-induced increase in activity (O'Brien et al., 1978; Thompson, 1983; Nelson et al., 1993). However, studies on the development of visual connections indicated the importance of a more subtle aspect of activity, the firing correlation between pre- and post-synaptic cells and between pre-synaptic inputs (Hubel and Wiesel, 1965), similarly to what previously suggested by Hebb for learning (Hebb, 1949). That similar mechanisms may underlie synapse elimination at the NMJ was clearly outlined (Purves and Lichtman, 1980), but direct experimental evidence has been lacking until recently.

One approach is to see whether inactivated inputs win or loose over active ones: evidence appeared supporting the first (Callaway et al., 1987) or the second alternative (Ribchester and Taxt, 1983; Ridge and Betz, 1984; Balice-Gordon and Lichtman, 1994), with final resolution in favor of the latter (Buffelli et al., 2003). A closer approach to the physiology of synapse elimination is to alter the correlation of firing in the presynaptic inputs: with *in vivo* experiments in which the competing motor terminals were all kept active but fired synchronously due to electrical stimulation, we found that synapse elimination is profoundly inhibited (Busetto et al., 2000). We used an adult model of synapse elimination, the reinnervation by a foreign nerve of an ectopic region of the rat soleus muscle: this entirely reproduces embryonic synaptogenesis (see Busetto et al., 2000) except for the distributed synapses that characterize

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Abbreviations: ABs, antibodies; αBTX, α-bungarotoxin; AChRs, acetylcholine receptors; CP, common peroneal; EDL, extensor digitorum longus; EPP, endplate potential; F-CSA, fiber cross-sectional area; HB, hybridoma; MEPPs, miniature endplate potentials; NMJ, neuromuscular junction; PT, posterior tibial; RMP, resting membrane potential; SEM, standard error of the mean; TTX, tetrodotoxin.

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the preparation. This important difference with normal development may raise objections that are addressed by the present study: in fact, it is also an adult reinnervation model, however of the original endplates, in which the multiple inputs converge on the same acetylcholine receptor aggregate, as in physiological NMJ formation.

EXPERIMENTAL PROCEDURES

Animals and surgical procedures

Experiments were carried out on adult male Wistar rats (180–350 g bw, Charles River, Calco, LC, Italy) and were authorized by the Istituto Superiore di Sanita' and the Ministry of Health of Italy. Surgery was performed under ether pre-anesthesia and equithesin anesthesia (9.7 mg/ml sodium pentobarbital, Sigma, Milano, Italy, and 42.5 mg/ml chloral hydrate, Carlo Erba, Milano, Italy: of this, 200–400 μl/100 g bw, i.p.). Soleus or extensor digitorum longus (EDL) nerves were crushed bilaterally, close to the nerve-muscle entry point, and left to regenerate and re-innervate the muscles *in vivo* for various time periods (2–3 weeks). On the day of the acute experiment the muscles were dissected out with their nerves for electrophysiological and morphological examinations. At the time of the initial crush, chronic sciatic nerve conduction block (using tetrodotoxin, TTX, Sigma, Milano, Italy) and nerve electrical stimulation distal to the block were also established, on one side only (see ahead for details). Controls were contralateral muscles in which only crush was applied and thus axonal regeneration occurred under natural impulse activity of motoneurons. In preliminary experiments, observing under the dissecting microscope the contraction of the soleus and EDL muscles evoked by nerve stimulation, we established that re-innervation starts 3–4 days after the crush. This confirmed previously published data (Buffelli et al., 1997). The duration of chronic stimulation *in vivo* was 13–17 days (mean 15) in one group of rats and 18–22 (mean 20) days in another. In a further series of reinnervation experiments we set up a nerve conduction block, without distal electrical stimulation, in order to investigate the effects of inactivity alone on synapse elimination.

Still in another group of rats (named pre-block) we established a conduction block as usual but delayed by 9–15 days (mean 13) nerve crush and onset of chronic stimulation: a further period of 9–15 days (mean 13) then elapsed before the final acute electrophysiological experiment. A variation of this protocol was to place the crush at the same time of the conduction block, start the stimulation several days later (11), and then carry it for 8–9 additional days before the acute experiment. The purpose of these two similar protocols was to investigate the role of inactivity as an enhancer of polyneuronal innervation. The data obtained with the two series were comparable and are lumped together. The appropriate controls were muscles in which axons regenerated during natural impulse activity following recovery of nerve conduction after the desired initial period of block had elapsed. To this end, the cuff surrounding the sciatic nerve was removed under a short lasting general anaesthesia as above: recovery of conduction occurred in about 1 and a half days and was signalled by the disappearance of the behavioural leg paralysis. The timing of cuff removal was such as to obtain approximately the same block duration as in the experimental group. The control experiments had to be done on different rats with respect to the experimental ones, because the same rat could not withstand a bilateral sciatic nerve block due to TTX toxicity. Also, for the experimental muscles separate experiments were done for EDL and soleus, as usual, for necessities imposed by the stimulation, while for the control ones EDL and soleus were from the same animals.

TTX conduction block

The sciatic nerve was freed from surrounding tissues for ~1.5 cm, inserted in a custom made silicone cuff (9 mm length, 1.4/5.0 mm ID/OD; MED-6382, NuSil Technology, Carpinteria, CA, USA) and continuously perfused with a TTX solution in saline (7.2–8.4 mg/day), from an Alzet osmotic pump (2ML4 or 2002; Alzet, Durect Corporation, Cupertino, CA, USA) implanted s.c., through a tubing. We previously showed that this procedure completely blocks conduction, avoids nerve damage and does not interfere with axonal transport or trophic functions of motor terminals (Pasino et al., 1996).

Chronic electrical stimulation

For chronic stimulation of nerves, we inserted either the common peroneal (CP) (EDL muscle experiments) or the posterior tibial (PT) nerves (soleus muscle experiments), into silicone cuff electrodes (3–4 mm long; 0.8/1.5 mm ID/OD for CP, 1.1/1.8 mm for PT). The bare ends of two multi-stranded steel wires, anode and cathode, slightly protruded into the cuff, with inter-electrode distance of 2 mm (AS-632, Cooner Wire, Chatsworth, CA, USA). The wires, led s.c. to the animal's back, reached the stimulator inside a tether, which was secured to the animal through a jacket. The rats were free to move in their cages, being connected to the stimulator through electrical swivels (Chatam, Hawthorne, CA, USA). To control whether the cuff electrodes could mechanically damage the nerves, we made: (i) clinical observations during the chronic experiment, and (ii) recordings of muscle contraction (see ahead) at the final acute experiment. First, we observed that the stimulation distal to the block always evoked a brisk and ample movement of the foot (only EDL or soleus nerves being crushed). Second, strength of contraction and muscle weight indicated that innervation and nerve-evoked activity were comparable in legs with or without cuff electrode (Results).

After surgery, the nerve conduction block developed within several hours. We then started the chronic stimulation with the following paradigm: trains of eight pulses (each pulse 100 μs in duration); one train every 8 s; total of 86,400 pulses/day. Up to the 9th day included, the pulse frequency in the train was kept at 15 and 10 Hz (for CP and PT nerve, i.e. EDL and soleus muscle experiments, respectively), then was increased to 80 and 20 (occasionally 30) Hz, respectively; train duration thus varied depending on frequency (88–700 ms). The lower frequency was used in the initial part of the stimulation protocol to avoid fatigue in synaptic transmission at newly formed neuromuscular junctions (Busetto et al., 2000). The pulse voltage was set at the onset of stimulation and checked thereafter several times a day to insure that the stimulus strength remained supramaximal for motor axons at all times. Using criteria established in a previous paper (Busetto et al., 2000), we determined each time the threshold for dorsal foot flexion (when stimulating the CP nerve) or plantar foot flexion (when stimulating the PT nerve) as indicators of threshold for evoked contraction of EDL or soleus muscles respectively; we then used a 3× foot threshold intensity as a supramaximal stimulus for CP or PT motor axons. Higher intensities were not used to avoid stimulation of pain afferents (Busetto et al., 2000), even though all stimulated nerves had a centrally located TTX block: animals never manifested behavioral indications of pain.

Electrophysiology

When the chronic *in vivo* treatment was over, the rats were anesthetized (equithesin) and EDL or soleus muscles isolated except for proximal tendon, nerve and vascular supply. The distal tendon was connected to a force transducer (Grass FT03E, West Warwick, RI, USA) for isometric recording of maximal twitch and tetanic contractions evoked by nerve stimulation, using the im-