PERSPECTIVE

Lesson from the neuromuscular junction: role of pattern and timing of nerve activity in synaptic development

The anatomical plan of adult muscle innervation is relatively simple: a given muscle comprises several motor units, each constituted by one motor neuron and the muscle fibers that it innervates; moreover, every muscle fiber is innervated by only one axonal terminal. In other words, motor units have separate, although intermingled, territories of innervation (**Figure 1D**). In striking contrast, the anatomical organization is different at birth, when every muscle fiber is innervated by several nerve terminals belonging to different motor neurons, a condition known as "polyneuronal innervation", with the consequence that motor units have larger and overlapped territories of innervation (**Figure 1A**) (Tapia and Lichtman, 2012). Soon after birth, redundant nerve terminals are progressively eliminated in a couple of weeks in rodents, and muscle fibers acquire their mature mononeuronal innervation. The same process occurs again in the adult muscle during reinnervation after nerve damage, when a transient period of polyneuronal innervation involves a good fraction of the fibers (Rich and Lichtman, 1989; Favero et al., 2010). At present, two major functional aspects of synapse elimination have been determined: 1) it is based on a competitive process between the multiple nerve terminals; 2) it is governed by neuromuscular electric activity, in particular by the relative pattern of action potentials between axon terminals competing for the same junction, which is the focus of the present article. The best evidence for competition is that the elimination process invariably brings to single axonal innervation and that no neuromuscular junction (NMJ) ever remains denervated, not even transiently before being eventually reinnervated by collateral sprouting.

The central role of electric activity in synapse development stems from classical studies of muscle paralysis or nerve stimulation, in which activity was either completely abolished or broadly increased, and resulted in the prevention or acceleration of synapse elimination, respectively (Tapia and Lichtman, 2012). Although providing clearcut results, these experiments have pushed neuromuscular activity to its extremes, whereas in the physiology of development all axons are active within a normal range. In particular, during muscle paralysis the block (or reduction) of activity is similar to the condition present *in utero* days before the onset of the competition/elimination process, namely before the first innervation occurs, but also during the early stages of neuromuscular development, when motor neurons and synapses are functionally immature and the total amount of activity transmitted to muscle fibers is very low. In these contexts, absent or low activity favors the expression of pro-innervation factors that trigger axonal growth, with the obvious goal of promoting innervation. Inactivity influences only the beginning of the NMJ development whereas during the later stage of synapse competition, all axons are active. Thus, to better investigate the competition/elimination process, experimental conditions should be utilized in which competing axons may differ in their pattern of activation but maintain physiological amounts of activity.

One important aspect about the pattern of activity is the relative timing of action potentials between converging inputs that may fire either at the same time (synchronous firing of spike *vs.* spike on a millisecond time scale) or without any temporal correlation (asynchronous firing). This issue has been first addressed by Hubel and Wiesel with breakthrough experiments on the development of binocular innervation of neurons of primary visual cortex in kittens: artificial squint resulted in monocular innervation of neurons that are binocularly innervated in normal development (Hubel and Wiesel, 1965). The interpretation of their result was that strabismus causes homolog portions of the two retinas to see different parts of the visual scene. The consequent asynchronous firing of output neurons drives the elimination of one of the two inputs. Extending these data to the development of the NMJ, we hypothesized that synchronicity of axonal activity allows the initial polyneuronal innervation of muscle fibers to occur, whereas asynchronicity drives synapse competition and elimination.

We first investigated the effect of synchronous axonal activity on polyneuronal innervation of muscle fibers during adult rat muscle reinnervation *in vivo* (Busetto et al., 2000). A "foreign" nerve (fibular) was bilaterally transposed to the surface of the soleus muscle in a region free of any acetylcholine receptors aggregate. After the original nerve was cut, the fibular axons quickly reinnervated the muscle fibers inducing *de novo* formation of acetylcholine receptors aggregates which were transiently polyneuronally innervated. During the reinnervation process, the action potentials in all fibular motor axons of the experimental side were made to fire synchronously by means of a combination of chronic block of spontaneous activity in the sciatic nerve and of electrical nerve stimulation distal to the block. The contralateral side was left unperturbed (natural activity) and served as a control. At different time points of reinnervation *in vivo*, we measured electrophysiologically *in vitro* the percentage of muscle fibers polyneuronally reinnervated (examples of a poly- and of a mono-neuronally innervated fiber in **Figure 1B** and **E**, upper panels, respectively): while control muscles showed a constant decline from a maximum of 20% toward values close to zero (one month after original nerve section), the experimental muscles showed quite higher (60%) and almost constant levels of polyneuronal innervation at any investigated time point. The interpretation of this result is that synapse competition and elimination are prevented when the action potentials are elicited synchronously within the competing axons. Muscles reinnervated *in vivo* at the original synaptic sites by their own nerves after crush, gave the same result (Favero et al., 2010).

We obtained the completion of this line of investigation with the challenging demonstration that asynchrony is the aspect of activity that physiologically drives synapse competition and leads to the development of mononeuronal innervation. For this purpose we selected a peculiar rat strain whose soleus muscle is innervated by two nerves (unlike the single nerve of normal muscles), that we could stimulate independently during reinnervation *in vivo*. The result was clearcut: when action potentials were evoked asynchronously between the two sets of axons, the competition/elimination process resulted as rapid and effective as in control muscles (Favero et al., 2012). It must be stressed that in this

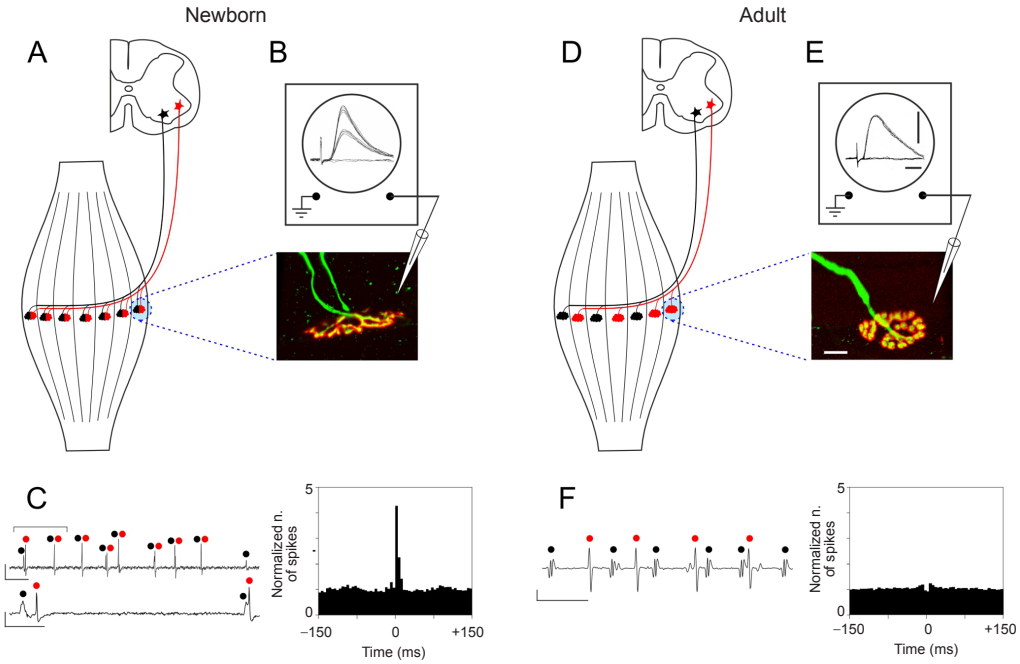

Figure 1 Polyneuronal innervation of muscle fibers and synchronous activity of motor neurons at birth
(A, D) Schematic of two indicative motor units: at birth the nerve terminals converge on the same group of muscle fibers, resulting in their polyneuronal innervation (A); after maturation their territories are segregated and the muscle fibers are mononeuronally innervated (D). (B, E) Schematic of electrophysiological (upper panels) and morphological (lower panels) analysis of poly- and mono-neuronal innervation, using intracellular recording and confocal fluorescence microscopy, respectively. Upper panels: synaptic endplate potentials (EPP; muscle action potentials blocked by curare) evoked *in vitro* by graded nerve electrical stimulation, showing a double EPP step indicative of a muscle fiber innervated by two distinct motor neurons (B), and a single EPP step indicative of a monoinnervated fiber (E). Lower panels: confocal images of a polyneuronally (B) and a mononeuronally (E) innervated fiber. Green: axons; red: acetylcholine receptors (muscle fibers not visualized). (C, F) Left: *in vivo* electromyographic recordings of motor units potentials from soleus muscles of a 3 (C) and a 29 (F) days old rat (different animals). In both cases two distinct waveforms are visible and marked with red or black dots (in F a third, small waveform is visible but not marked; in C the lower trace is the expansion of the portion in bracket). Note that at birth the 2 motor units are always active at the same time (C, synchrony), whereas in the adult their firing is completely uncorrelated (F, asynchrony). (C, F) Right: averaged cross-correlograms of all motor units pairs recorded a few days after birth (C, 30 pairs, embryonic day 21 through postnatal day 5) and in the adult (F, 47 pairs, postnatal day 13 through 30). Time zero marks the occurrence of the motor unit taken in each couple as reference. A peak near time zero indicates a significant probability of the measured motor unit to be active synchronously with its reference unit. Number of events are normalized to the mean number of events outside the peak. Scale bars: (B, E) EPPs: 2 ms/mV. Confocal images: 10 μm. (C, F) Time: 100 ms (C upper trace), 25 ms (C lower trace and F). Voltage: 200 μV. (C, F) Data from Buffelli et al. (2002).

experiment the two nerves received the same amount of activity and differed only in their timing of activation. Equally important, by varying the degree of asynchrony we identified a time window of 25 ms within which action potentials of competing inputs in a polyneuronally innervated NMJ are sensed as synchronous and competition is prevented. We will return on these two points later.

Several other experiments have also demonstrated that a difference in amount of activity favors synapse competition. Particularly informative is an experiment performed in developing mice in which a subset of motor neurons was made inactive by disruption of the gene for choline acetyltransferase: the inactive axonal terminals were always eliminated when competing against active terminals (Buffelli et al., 2003), supporting the conclusion that the terminals more active or possessing more efficient synapses, are those that ultimately win the competition (Tapia and Lichtman, 2012). The terminal Schwann cell may play a role in this battle, given its ability to detect functional differences among converging terminals (Darabid et al. 2013) and to induce rapid modification of synaptic efficacy based on activity (Darabid et al. 2014). The involvement of the terminal Schwann cell is further elucidated by studying the mechanism of nerve terminal retraction in animal models of neurodegenerative diseases such as amyotrophic lateral sclerosis (Pollari et al., 2014; Arbour et al., 2015). An explanation of competition based on different amount of activity is not alternative to but (may) actually cooperate(s) with the mechanism based on differential timing of activation (*i.e.*, synchronous *vs.* asynchronous) demonstrated by us. What in fact they have in common is that competition is activated whenever of two competing inputs, one is firing at the time the other is silent: but the "timing" mechanism is the primary one because, as explained above, we obtained powerful competition and elimination between converging axon terminals that were made asynchronously active *but with the same amount of total activity.*

The robust effect of artificially-induced asynchronous activity (and natural activity as well) in promoting synapse competition, prompted us to examine a further question: how could the initial polyneuronal innervation ever occur, given the fact that motor neurons are known to fire individual action potentials independently, *i.e.*, asynchronously to each other? A possible solution is to hypothesize that motor neuron firing is initially synchronous in character during the period of polyneuronal innervation, to become asynchronous only after birth. To test this, we recorded simultaneously the natural activity of different motor units of a given pool in awake rats (Buffelli et al., 2002). We found it

to be highly correlated during a few days after birth: in other words, motor neurons tend to fire their action potentials synchronously (**Figure 1C**). In the following days the firing quickly became uncorrelated and this occurred before the beginning of synapse competition and elimination (**Figure 1F**). During the same period the amount of activity of single motor units, initially very low, significantly increased. Finally, the time window of synchronization (width of the peak of cross correlation) was ~25 ms, that well corresponds to the time window within which the action potentials are sensed as synchronous by polyneuronally innervated NMJs, as described above. Suggestions for the mechanism of motor neuron synchronization at early stages of development are: 1) gap junctions providing electric connections between adjacent motor neurons, or 2) a common excitatory drive impinging on motor neurons.

One important question is still unanswered: which are the molecular messengers involved in the competition/elimination process? Several candidates have been proposed to mediate competition, some of them acting as punishing factors [*i.e.*, promoting synapse elimination: glia cell line-derived neurotrophic factor (GDNF) and pro-brain-derived neurotrophic factor (pro-BDNF)], others acting as rewarding factors (*i.e.*, preventing synapse elimination: mature-BDNF) (Darabid et al., 2014). Because of the instructive role of pattern of activity, in order to determine if a given molecule/cell mediates competition, its relation to the timing of activity must be investigated: for none of them this has yet been done.

Recently an alternative view has been proposed, essentially that synaptic elimination is not an activity-dependent but rather a random process, eventually leading to mononeuronal innervation (Turney and Lichtman, 2012). This hypothesis is based on vital time-lapse imaging of newborn multiply innervated NMJs describing in detail the rapid changes of single muscle fiber innervation by converging axonal terminals: the authors tested the efficiency of small terminals to take over denervated portions of the synapse after focal damage of the competing inputs, and concluded that during normal development, the retraction of redundant inputs is a primary and random event. Unfortunately this proposed mechanism is based only on morphological observations, and no functional experiments have been performed to prove them. Moreover, its relationship to the timing of activity has not been explored.

To summarize, based on the experimental data we propose the following scenario: motor neurons around birth have a low level of activity and especially a synchronous type of firing, conditions that, respectively, allow axonal growth and favor the transient expression of polyneuronal innervation of muscle fibers. Soon after birth, their activity pattern becomes asynchronous and its total amount increases, thus inhibiting axonal growth and favoring synapse competition and elimination of all but one terminal (see "Discussion" in Favero et al., 2012, 2014). It is important to recall that redundant innervation is a common developmental feature also throughout the central nervous system, and that activity plays an instructive role in its pruning (Tapia and Lichtman, 2012). More specifically related to the synchrony/asynchrony developmental paradigm, a recent study performed in mouse pups *in vivo* has demonstrated that asynchronous photic activation of the eyes obtained desegregation (*i.e.*, synaptic competition and elimination) of inputs in the superior colliculus and the lateral geniculate nucleus, synchronous eyes activation and segregation of inputs being the normal feature in these central visual targets (Favero et al., 2014; Zhang et al., 2012). In conclusion, the neuromuscular junction, besides being the preparation were polyneuronal innervation and synapse elimination have been first described, is also one of primary election for the functional and mechanistic study of these phenomena and relevant for the understanding of the development of the entire brain.

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