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Axonal competition and synapse elimination during neuromuscular junction development

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Abstract

Early in development, each muscle fiber is innervated by multiple neurons. Different activities between nerve terminals on the same junction cause the elimination of all but one of them through Hebbian competition. The molecular basis of this developmental synapse modulation is still being investigated because it is a model for how the nervous system achieves the proper synaptic connectivity and function. The study of the vertebrate neuromuscular junction has proven useful to understand the carefully regulated process of axon withdrawal, locally regulated in the synaptic site by the nerve terminals themselves, the postsynaptic muscle cell and the Schwann cells. In this review, we summarize the multiple factors that have been implicated in axonal competition and synapse elimination.

Key words: postnatal synapse elimination, axonal competition, neuromuscular junction polyneuronal innervation.

Introduction

Proper synaptic connectivity is essential for the precise function of the nervous system. The well-orchestrated synaptic circuits present in the adulthood have been rearranged during development to achieve the complex and accurate neural function. During mammalian development, several neurons contact with a target cell and make multiple synaptic connections although most of them disappear during early postnatal life. The synaptic pruning process takes place without neuron death [1]. This physiological phenomenon was first described at the neuromuscular junction (NMJ) in mammals [2] and thereafter it has been observed in many parts of the central nervous system [3]. Hebbian competition between axons with different activities leads to the loss of roughly half of the synapses initially produced, so connectivity is refined and specificity gained.

The vertebrate NMJ is a useful model for studying the synaptic elimination mechanism, which includes elimination and remodeling of existing synapses during the initial postnatal development. At birth, each skeletal muscle cell is innervated by several motoneurons but, in a short period of time (a few weeks in rodents), all but one of those axonal branches are withdrawn (figure 1). This loss of synapses and axons is thought to be based in a competitive process between axons innervating the same muscle fiber [1,4]. It has been demonstrated that synaptic activity is essential to initiate synapse loss and the mechanism appears to depend on the relative activity patterns of the competing inputs [5,6], with the more active input gaining synaptic territory and the less active input retracting [7–10].

What are the signals involved in the synapse elimination process? Many studies have described the detailed sequence of the withdrawal mechanism. However, although several regulatory factors have been involved, it remains unknown how do they articulate to regulate synaptic competition and elimination during development.

Axon withdrawal is a carefully regulated process

At birth, the branches of different inputs, placed at the same NMJ, intermingle and occupy areas nearly equal. At the functional level, these inputs have similar strengths [11] but their fate is not yet determined and requires the subsequent competitive mechanism that causes synapse loss. From this moment, the surplus of synaptic connections is removed in a carefully regulated way. Observing the dynamic of the elimination process (mainly by *in vivo* fluorescent time lapse imaging), the axonal branches in process of withdrawing are shown to suffer distal-to-proximal retraction (accompanied by the formation of a retraction bulb) without degeneration and are resorbed into the parent axon [12–14]. The gradually vacated contact sites are mostly reoccupied by the axon that ultimately remains, increasing thus its synaptic area [14,15]. The location changes between competing axons occur in parallel with the acquisition of different synaptic strength, which precedes the withdrawal of the weaker input/s [11]. However, sometimes, a "flip-flop" mechanism is observed, in which inputs initially occupying larger spaces finally retreat and vice versa [14], indicating that axons are highly dynamic.

Over the first several postnatal weeks, the number of junctions that are polyinnervated gradually declines, showing temporal differences between different muscles [16,17]. Moreover, elimination is also asynchronous between fibers belonging to the same muscle, with some NMJs remaining polyinnervated while other junctions are monoinnervated [17]. This withdrawal is asynchronous in another sense: different collateral axons of the same motorneuron innervating several fibers can be observed, at a particular time, at different stages of withdrawal [12]. This evidence indicates that synapse elimination is locally regulated at each NMJ site, probably with the involvement of the postsynaptic cell and the terminal Schwann cells in the process.

Postsynaptic cell as intermediary

The distribution of acetylcholine receptors (AChR) within and around the neuromuscular junction changes dramatically during the first postnatal weeks at the same time polyneuronal innervation is being eliminated. Axon withdrawal at each NMJ is contemporaneous with and may be related to the transformation of the postsynaptic AChR cluster from a unique plaque-shaped site where multiple axons converge to the branched pattern seen some weeks later [18–21]. This evidence reinforces that the postsynaptic cell may be intermediary in axonal competition.

A careful examination of the time course of loss of innervation and the disappearance of postsynaptic receptors reveals that there is a degree of independence between these two processes [20–22]. A considerable decrement in polyneuronal innervation occurs at a time when relatively little loss of the postsynaptic AChR can be demonstrated [20]. On the other hand, local receptor loss has been observed before the corresponding axon loss [18,23]. This outcome maintains the idea that changes in the postsynaptic cell may occur before the axon has withdrawn and implies that pre- and postsynaptic changes in developmental synapse elimination are closely coordinated.

Terminal Schwann cells as intermediary

Non-myelinating terminal Schwann cells (tSC) at the NMJ play a role in synapse elimination (reviewed by [24]). At birth, the several tSCs present at each endplate branch are interdigitate extensively without delimiting territories, as they do at mature junctions.

Their processes separate nerve terminals from each other and can directly appose AChRs in the muscle cell membrane [25,26]. A detailed electron microscopic study showed that tSC contribute to synapse elimination by performing a random disconnection of the converging immature axons from the muscle fiber and even attacking them phagocytically [26]. This evidence suggests a model in which the activity of tSCs may promote synapse elimination by creating vacant synaptic sites that can be reoccuppied by the competing terminals [24]. It has been described that a factor that controls the participation of tSCs in the synapse loss process is a motor axon-tethered isoform of neuregulin1 (NRG1-III) [27] contributing, thus, to the multifactorial regulation of the process. However, not only tSCs are involved in the synapse loss mechanism. The non-terminal myelinating SCs are related to the glial isoform of neurofascin (Nfasc155) [28] and the Grb2-associated binder 1 (Gab1), the latter required for NRG1-induced peripheral nerve myelination [29], and both molecules are necessary for postnatal remodeling of synaptic circuitry.

Activity-dependence of synapse elimination

Synapse elimination has been extensively described as an activity-dependent process (reviewed by [10,30]). It is inhibited by the block of neuromuscular activity and it accelerates by through nerve electrical stimulation [31]. However, asynchronous activation of the postsynaptic cell could be a fundamental feature to determine which axon wins and which loses [5,6,10,32–34]. Hebbian competition between nerve endings with different activities would result in the elimination of the less active input and the gain of synaptic territory of the more active input [7–10,35].

The NMJ is a cholinergic synapse and it has been proposed that acetylcholine mediates this activity-dependent process [36]. However, glutamatergic transmission is also present at the NMJ [37,38]. In particular, glutamate is derived from the nerve terminal whereas NMDA receptors are postsynaptic. It has been determined that NMDA signaling in the NMJ enhances synapse elimination during the first two postnatal weeks [39]. Therefore, the activity-dependent process of synapse loss might have to coordinate cholinergic and glutamatergic neurotransmission. In addition, other metabotropic receptors activated in the context of synaptic activity also play a role in synapse elimination (see later; [22,40]). However, other evidence showed that activity is not decisive on the synapse elimination process [41–43]. Therefore, synapse elimination, although controlled by different activity levels between competing motoneurons, is a complex mechanism that depends on multiple factors.

Molecular factors involved in synapse elimination

The cell mechanism leading to the massive circuit changes occurring during postnatal development is not fully known as well as its molecular drivers. It was first suggested that endogenous calcium-dependent proteases released within the synapse may be involved in nerve terminal destabilization [44–46]. Specifically, the naturally occurring serine protease thrombin mediates the activity-dependent synapse loss at the NMJ [44,47,48], probably by destabilizing AChR clusters [49]. Moreover, thrombin may affect synapse elimination via protein kinase C activity [33,48]. The serine-threonine protein kinases A (PKA) and C (PKC) have been extensively involved as mediators of the selective activity-dependent synapse reduction *vs* stabilization [20,34,50]. In particular, PKC activation destabilizes synapses whereas PKA stabilizes them [51]. Activation of PKA and PKC have opposite effects on AChR stability and their balance

may play some role in synapse loss [52]. In fact, it has been hypothesized a kinase-based model for Hebbian synapse loss based in spatially specific and opposing actions of the PKCθ isoform and PKA. A localized positive effect of activating a given input to the muscle (mediated by PKA), can neutralize the general, negative, synapse-eliminating effect (mediated by PKC) resulting from the input activation. These effects may result in activity-dependent alterations of synaptic connectivity at both the nerve inputs and the postsynaptic nAChR clusters [51,53]. In fact, several metabotropic receptors present in the NMJ (mainly neurotrophic, muscarinic and purinergic receptors, see later) that have been related to synapse elimination [22,54,55] converge on PKC and PKA [56].

It has been proposed that active synaptic sites can destabilize inactive synapses in their vicinity by competing for a trophic factor secreted by the postsynaptic cell in limited supply [57,58]. The various neurotrophins expressed in skeletal muscle [brain-derived neurotrophic factor (BDNF), neurotrophin (NT)-4, and NT-3] mediate their effects by binding to two types of cell surface receptors in the motor nerve terminals, $p75^{NTR}$ receptor and a related family of tyrosine protein kinase receptors (Trks). The neurotrophins and their receptors are involved in both ACh release and axonal retraction during postnatal axonal competition and loss [40,59,60]. Their signaling pathways have a different spatial and temporal expression during development and thus may contribute differently to the synapse loss [61–64]. It has been described a model in which pro-BDNF and mature mBDNF serve as potential "punishment" and "reward" signals for inactive and active terminals, respectively [60]. In relation with this, the blockade of the BDNF-receptor trkB during synapse elimination, results in an initial delay (suggesting increased but unresolved competition) finally followed by an acceleration of axon loss [59]. Also, NT-3 is involved in the developmental mechanism that eliminates redundant synapses [63]. Ciliary neurotrophic factor (CNTF) promotes the retention of polyneuronal innervation of developing skeletal muscle fibers. Glial cell line-derived neurotrophic factor (GDNF) has an important effect on synapse elimination [12] although it does not acutely modulate transmitter release during the developmental process of synapse elimination [62].

There is evidence indicating that, in addition to trkB, presynaptic autoreceptors (muscarinic acetylcholine receptors –mAChR- and adenosine receptors –AR-) play an important role by allowing the nerve terminals to communicate directly during the competition in the NMJ [22,40,56,65]. mAChR may mediate the direct competitive interaction between nerve endings because their different activity-dependent acetylcholine (ACh) release [55,66,67]. Therefore, the more active endings may punish the less active ones or reward themselves [40]. However, as stated, not only differences in the amount of activity between competing terminals but also their timing are important because asynchronous activity promotes synapse elimination whereas synchronous activity prevents it [10]. The weakest nerve endings (those that evoke endplate potentials (EPP) with the least quantal content) in polyinnervated junctions seem to have an ACh release inhibition mechanism, based on muscarinic autoreceptors coupled to PKC and voltage-dependent calcium channels (VDCC), which can depress the ACh release capacity in these endings and may contribute to functionally disconnect them [55,66–68]. In this context, it has been showed that the cooperation of M_1, M_2 and M_4 muscarinic subtypes favors axonal competition at the end of the first postnatal week and promotes the full sequence of axonal loss and synapse elimination shortly thereafter [22].

However, an axon that fails and is eliminated from one NMJ can win the competition at another [12], which suggests the importance of local effectiveness with the involvement of other signaling pathways and postsynaptic muscle cell-derived

factors. At least, trkB signaling plays this role and cooperates with muscarinic signaling favoring synapse elimination [22,59,66]. In addition, ATP and adenosine release promotes stabilization of the neuromuscular junction and may play a role in activity-dependent synaptic modification during development [69]. In particular, adenosine receptors (A_1 and A_{2A}) are involved and cooperate with other signaling [54]. Recently, it has been identified an unexpected role for the major histocompatibility complex class I (MHCI) in the elimination of neuromuscular synapses during development [70] extending thus the factors involved in the process. Specifically, developmental synapse elimination is promoted by specific immune proteins, members of MHCI.

Several studies have provided fundamental insights into among the importance of the temporal activation of several molecular factors to achieve the naturally occurring synaptic remodeling during the postnatal development (70, 34, 39 22, 40). This evidence indicates that between the first and second postnatal weeks there is a crucial period for synapse elimination.

It is evident that neuromuscular developmental synaptic elimination is a multifactorial process in which most molecular changes are related with changes in signaling pathways that communicate the synaptic cells. The phosphorylating activity of kinases, as PKC and PKA, on exocytotic proteins in the axons, and, in the postsynaptic receptors may be important in modulating synaptic functionality and, therefore, stability during the process.

Conclusion

Developmental activity-dependent Hebbian synaptic plasticity is of central interest for neuroscience. The postnatal synaptic elimination is essential to achieve the proper synaptic connectivity and function of the nervous system. The study of the vertebrate NMJ has been useful to increase the knowledge about the carefully regulated process of axon withdrawal, locally regulated and in which postsynaptic muscle cell and the tSC contribute. Synapse elimination, although controlled by different activity levels between competing motoneurons, is a complex mechanism that depends on multiple factors related with several signaling. Finally, the advances in this field will be of great interest to determine which molecular pathways are shared by the different forms of nervous system plasticity as developmental synapse elimination, learning, aging, injury and disease adaptation, and regeneration.

Legend of the Figure 1.

Synapse elimination removes redundant connections to transform the newborn polyinnervated system into the adult monoinnervated neuromuscular junctions.

A. Circuitry editing depends mostly on synaptic activity and is a highly dynamic local process controlled by multiple factors and in which the postsynaptic cell and the terminal Schwann cell are involved.

(a) At birth the branches of different inputs, placed at the same AChR oval postsynaptic plaque-shaped site, intermingle and the areas occupied are nearly equal. These inputs have similar strengths. Several tSCs are present at each NMJ and interdigitate extensively.

(b) From this moment, the surplus of synaptic connections is removed in a carefully regulated way. The axonal inputs are eliminated by gradually vacating their synaptic contact sites and the axon that remains gradually occupies many of the synaptic sites that were previously occupied by the lost axons. In some cases, inputs initially occupying large areas can begin to retreat and smaller terminals take over the vacated space (*). Retraction bulbs form at the end of retreating axonal branches (**), which are reabsorbed into the parent axon. The postsynaptic site develops one or more AChR-free areas that are non-innervated. The competing axons acquire different synaptic strength, which precedes the withdrawal of the weaker input/s.

(c) Finally, all but one inputs are eliminated and each muscle fiber is innervated in its branched AChR endplate by only one input.

B. Confocal image showing several NMJs at postnatal day 9 immunostained in green with neurofilament-200 and in red with rodamine-alpha-bungarotoxin. Several degrees of polyinnervation are observed. *monoinnervated, **doubly and ***triply innervated NMJs. Arrow indicates a retraction bulb. Scale bar: 10 μm.

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