Neuromuscular synaptogenesis: coordinating partners with multiple functions

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Abstract | The formation of highly efficient and reliable synapses at the neuromuscular junction (NMJ) relies on dynamic molecular interactions. Studies of the development and maturation of the NMJ have focused on events that are dependent on synaptic activity and that require the coordinated actions of nerve- and muscle-derived molecules with different targets and effects. More recently, perisynaptic Schwann cells — the glial cells at NMJs — have become an important focus of research. These glia concomitantly contribute to pre- and postsynaptic maturation while undergoing maturation themselves. Thus, an intricate 'danse à trois' regulates the maturation of the NMJ to form a highly efficient communication unit, in which fine glial processes lie in close proximity to a highly concentrated population of postsynaptic receptors and perfectly aligned presynaptic release sites.

Neuromuscular junction (NMJ). A unitary functional structure composed of a single axon terminal innervating a muscle fibre. The presynaptic terminal is covered by specialized glial cells called perisynaptic Schwann cells.

Perisynaptic Schwann cells (PSCs). Non-myelinating glial cells at the neuromuscular junction. They originate from the neural crest but differ structurally and phenotypically from axonal myelinating or axonal non-myelinating Schwann cells.

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During embryonic development in vertebrates, motor neuron axons grow long distances from the spinal cord to reach their distal targets[1]. They form the link between the CNS and the rest of the body — particularly the striated muscles that effect voluntary movements. The direction of this long-distance axonal travel towards specific muscles to be innervated requires well-orchestrated interactions among axons, axonal Schwann cells and environmental cues[1,2]. Owing to its large size, accessibility and simple organization, the neuromuscular junction (NMJ), which enables the nervous system to control the muscular system, has been used extensively to study synapse formation[3,4]. However, this apparent simplicity belies the complex processes that take place during synapse formation and that are necessary for NMJ function. Indeed, the formation of perfectly aligned presynaptic assemblies and postsynaptic compartments requires an intricate interplay between molecular signals. Ultimately, the two elements become tuned functionally to one another, with the level of presynaptic transmitter release adapted to the capacity of postsynaptic activation.

Synapse formation is followed by another major step, synapse maturation, which leads to the stabilization of all of the synaptic elements to form a reliable synapse. It has long been acknowledged that the maturations of pre- and postsynaptic elements at the NMJ are interdependent; however, until recently, the elaborate interactions between these two compartments were poorly understood. Presynaptic maturation was previously assumed to be controlled by molecules that are released by the postsynaptic apparatus, and vice versa[5–8]. However, recent advances in molecular analysis (for instance, improvements in fusion proteins and cell-specific gene deletion) have revealed a more complex scenario, wherein some molecules regulate both pre- and postsynaptic organization. Thus, it is possible that the formation and maturation of pre- and postsynaptic compartments are in fact tightly coordinated by shared molecular mechanisms. Moreover, although NMJ formation was originally thought to be regulated by only pre- and postsynaptic mechanisms, more recent evidence has implicated a role for glial molecular mechanisms in coordinating the maturation of the two synaptic elements. Indeed, it was reported that perisynaptic Schwann cells (PSCs; also known as terminal Schwann cells) produce and release synaptogenic factors[9–14], and their own maturation is modulated by pre- and postsynaptic molecules[15–20]. This tri-directional interaction is also required for key activity-dependent changes that are involved in NMJ maturation and is particularly important during synaptic competition and elimination. Indeed, PSCs detect activity from competing synapses[18] and are responsible for debris clearance and the elimination of superfluous nerve terminals[21,22]. Thus, PSCs could represent a link between the activity-dependent and molecularly regulated changes that are essential for the maturation of the NMJ.

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In this Review, we discuss the growing evidence of a tri-directional molecular and activity-dependent crosstalk among presynaptic terminals, postsynaptic muscle fibres and PSCs at the developing NMJ. This coordination structures and organizes the presynaptic release machinery and postsynaptic receptors, and thus enables the tight assembly of an efficient and reliable communication unit.

Molecular mechanisms of NMJ development

NMJ formation, which is initiated during embryonic development (around embryonic day 12 (E12) to E13.5 in rodents)[5,7,23,24], is preceded by the guidance of the axon towards the muscle fibre that is to be innervated and involves accurate molecular signalling to ensure the formation of precise nerve–muscle contacts. A period of maturation follows that leads to morphological and functional changes to the pre- and postsynaptic elements. Maturation of the NMJ depends on molecular mechanisms that elaborate the complex pre- and postsynaptic organization and that reduce the number of axonal terminals at a single NMJ until only one remains; these mechanisms are necessary for efficient synaptic communication. Presynaptic terminals must express synaptic proteins to form active zones, whereas postsynaptic elements express high densities of aggregated receptors at the crests of postsynaptic folds. Together, it is the 'synaptic elements' — namely, the presynaptic nerve terminal, the postsynaptic muscle fibre and the associated perisynaptic glia — that collectively coordinate the maturation of the NMJ.

Pathfinding, guidance and pre-patterning. During embryonic development, pools of motor neurons in the spinal cord form organized motor columns (already present by E6–7 in chick embryos)[25–27], which can be classified according to their location (rostrocaudal, mediolateral or dorsoventral) within the spinal cord[27,28]. This columnar organization seems to be crucial for establishing the proper inputs of each class of motor neuron to their specific effector targets to control complex locomotor behaviour[27,29,30]. This well-structured organization suggests that elaborate mechanisms are required to guide the right group of motor axons to their specific final target. It is now recognized that pathfinding is not controlled by the target muscle per se[31] but instead by guidance cues along the path of the motor neuron[27,32], and that this process is dependent on neuronal activity[33]. These mechanisms, and the wide range of genes and transcription factors involved, have been discussed elsewhere (reviewed in REFS 1,27).

When motor axons reach their final target (the timing of which varies according to the muscle and its location; around E12.5 for the mouse diaphragm)[23], muscle fibres already show a certain level of maturation, as indicated by the presence of clusters of ionotropic acetylcholine receptors (AChRs) on their surface[23,34,35]. The presence of AChR clusters on the fibres prior to the arrival of the motor axon indicates that these AChRs are not directly linked to synaptic activity. This has raised the question of whether the pre-patterning (that is, nerve-independent clustering) of AChRs dictates the location of NMJs on muscle fibres[23,34,36–38]. Although initial work in cell culture has shown that NMJs do not form preferentially at pre-existing AChR clusters[39,40], evidence obtained from mammals and other vertebrates suggests that AChR pre-patterns have a role in defining the location of nerve–muscle contacts[37,38,41,42].

Upon contact with muscles, motor axons innervate muscle fibres extensively to generate polyinnervated NMJs and large motor units[43–45]. Although NMJs are functional at this stage, the motor neurons are not efficient enough to induce muscle contractions in a refined and precise way; this efficiency requires NMJ maturation. Even though pre- and postsynaptic maturation are discussed separately below, the two processes are interdependent and involve molecules that are derived from, and act on, all three synaptic elements.

Postsynaptic maturation. The postsynaptic compartment undergoes major rearrangement during NMJ maturation, including increases in the number, stability, density and clustering of AChRs; these changes are mediated by key molecular mechanisms.

The proteoglycan agrin is the most studied molecule involved in postsynaptic organization. Mice that lack agrin (*Agrn*$^{-/-}$ mice) show major defects in postsynaptic AChR organization: AChRs in these animals are uniformly distributed on the surface of the muscle fibre and do not cluster after muscle innervation[46]. These mice also show presynaptic defects, such as poor arborization of motor axons[46] and aimless overgrowth of nerves[23]. However, in these animals, the pre-patterned AChR clusters that form prior to innervation are not affected[23,34,46]. Interestingly, intramuscular injection of recombinant agrin induces the formation of AChR clusters in denervated muscles and the formation of ectopic AChR clusters in adult muscles[47–49]. Agrin can be synthesized and released by neurons, muscle fibres and Schwann cells (hereafter defined as SCs when referring to Schwann cells that are associated with axons or when the type is unclear), and has varying AChR-clustering efficacy depending on alternative splicing of the agrin gene[50,51]. Generally, neuronal forms of agrin are more potent at clustering AChRs[11,52,53]. However, AChR clustering can be enhanced by the active PSC-derived form of agrin — for instance, at the end of tadpole metamorphosis or during nerve regeneration at the amphibian NMJ[11].

Agrin triggers the autophosphorylation of the muscle-specific tyrosine kinase receptor (MUSK)[54]. MUSK is enriched in the postsynaptic endplate, colocalizes with AChR[55] and has a pivotal role in AChR clustering. Mice lacking MUSK (*Musk*$^{-/-}$ mice) show a more severe phenotype than do *Agrn*$^{-/-}$ mice: *Musk*$^{-/-}$ mice show neither AChR pre-patterns nor nerve-induced clustering of AChRs, and their motor axons grow aimlessly over the muscle[54]. Agrin interacts with MUSK via low-density lipoprotein receptor-related protein 4 (LRP4), a transmembrane protein that is enriched at the NMJ[56–58]. Mice that lack this lipoprotein die at birth and show major defects that are comparable to those seen in *Musk*$^{-/-}$ mice[56]. Indeed, LRP4 links agrin and MUSK activation, and is required for AChR clustering[56–58]. Furthermore, the association of LRP4 with MUSK is sufficient to trigger MUSK

Active zones
Areas on the surface of the presynaptic terminal that are characterized by their electron-dense appearance owing to the high concentration of proteins involved in Ca^{2+}-dependent synaptic-vesicle exocytosis and recycling.

Motor columns
Groups of motor neurons that innervate selective sets of muscles.

Pre-patterning
A nerve-independent phenomenon that occurs prior to the arrival of motor axons whereby acetylcholine receptors cluster in the central region of the muscle fibres (along the longitudinal axis), purportedly defining the location of nerve–muscle contact.

AChR clustering
The gathering of acetylcholine receptors (AChRs), which is regulated by molecular mechanisms. It is one of the initial steps of synapse maturation.

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autophosphorylation — even in the absence of agrin — and LRP4 is important for the pre-patterning of AChR clusters prior to the arrival of the nerve terminal[57,58]. As a tyrosine kinase receptor, activated MUSK influences numerous downstream signalling pathways (reviewed in REF. 59) and promotes the accumulation of synaptic membrane proteins such as rapsyn and neuregulin receptors. As well as being a self-associating protein, rapsyn can cluster AChRs, dystroglycan and other important NMJ proteins. Mice that are null for rapsyn show diffusely distributed AChRs over the surface of the muscle fibre and have impaired neuromuscular transmission[60] (reviewed in REF. 8).

WNT ligands are secreted molecules that regulate many aspects of embryonic development, including cell survival, cell death, cell proliferation and axon guidance, AChR aggregation and AChR clustering[61,62], as well as the differentiation of the pre- and postsynaptic elements of the NMJ (reviewed in REFS 62,63). In zebrafish, WNT ligands interact with MUSK — before the nerve arrives at the NMJ — to induce the pre-patterning of AChRs on muscle fibres[7,64]. WNT proteins could subsequently participate in AChR clustering while nerve–muscle contacts form[65,66]. WNTs seem to be both positive and negative regulators of AChR clustering. In cultured mouse myotubes, WNT3, a WNT protein that is expressed by nerve terminals at the mouse NMJ, acts with agrin to increase AChR clustering[65]. By contrast, WNT3A, another isoform of WNT, suppresses the expression of rapsyn and thus inhibits AChR aggregation at the surface of cultured myotubes[66]. Moreover, WNT can be released by motor neurons and by other cell types, including glial cells[14,67]. In *Drosophila melanogaster*, Wingless proteins (which are WNT orthologues) from glia at the NMJ facilitate the clustering of glutamatergic postsynaptic receptors[14]. However, although the roles of WNT proteins in postsynaptic maturation are gradually being revealed, their *in vivo* contributions at the mammalian NMJ remain poorly understood owing to their diverse actions[62,63] and cellular sources[14,67].

The maturation of the NMJ must coincide with an increase in the synthesis of AChR and its associated proteins such as rapsyn and MUSK. Neuregulins — in particular, neuregulin 1 — that are expressed by motor neurons, muscle fibres and SCs[68–70] may regulate the transcription of AChR-encoding genes and functional receptor expression[70,71]. Indeed, NMJs do not form properly in mice that lack neuregulin 1 or its receptors, ERBB2 and ERBB3 (REFS 16,17,72). Extracellular neuregulin 1 binds to these tyrosine kinase receptors of the ERBB family to promote the synthesis of AChR and its associated proteins[68,71,73]. However, the exact signalling pathway that is involved in promoting AChR synthesis and clustering remains elusive, and studies have questioned whether neuregulins and their receptors are necessary for NMJ formation[15,74]. For instance, the specific ablation of muscle ERBB2 and ERBB4 receptors in mice did not result in major defects[15], whereas overexpression of a constitutively active form of the ERBB2 receptor in muscle fibres induced the aberrant formation of NMJs[75]. One study showed that motor-neuron-derived neuregulin 1 was not necessary for proper AChR density and clustering, and that muscle neuregulin 1 was not necessary for postsynaptic maturation[74]. The ambiguity associated with the role of neuregulin 1 may be partly due to the multiple cellular targets and functions of neuregulin 1, notably in SC survival and maturation[16,17,72,76]. Indeed, SCs are absent in mice that lack neuregulin 1, ERBB2 or ERBB3, and NMJs in these mice do not form properly and are not maintained[16,17,72]. As PSCs express neuregulins and their receptors[69], it has been proposed that the function of neuregulin 1 — in particular, its effect on postsynaptic maturation — may be mediated indirectly via PSCs[8,15] (rather than by its direct effects on the muscle fibre). If this is the case, then both neuronal and PSC-derived neuregulins could be important for the maturation and maintenance of the NMJ, as well as for the regulation of the synthesis of AChR and its associated proteins. It remains unclear whether the NMJ defects observed in mice that are null for neuregulin 1, ERBB2 or ERBB3 are due to the absence of SCs or the lack of neuregulin signalling, or both. Studies examining the effect of deleting PSC-derived neuregulin 1 or neuregulin 1 receptors at the surface of PSCs would help to resolve this issue. Nonetheless, it is apparent that a combination of molecules that are derived from various sources must act in a concerted way to regulate and promote AChR clustering, as well as postsynaptic assembly and maturation (TABLE 1).

Although most of the mechanisms described above promote the synthesis and clustering of AChRs, other molecular signals promote the removal of improperly localized AChRs and limit the number of AChRs. Interestingly, this regulation is partly activity-dependent, as neuronal ACh release inhibits the expression of AChRs and promotes their endocytosis[77,78]. NMJs that lack choline acetyltransferase (ChAT) — which exhibit very low levels of ACh synthesis — are larger, more complex and have a broader distribution of AChR clusters, which suggests that ACh antagonizes AChR clustering[78–80]. In fact, synaptic activity may trigger positive- or negative-feedback mechanisms depending on the state of postsynaptic AChRs. As the nerve–muscle contact is formed, nerve terminals release positive signals — such as agrin — that promote AChR clustering. However, if AChRs are not stabilized, then cholinergic neural activity promotes their disruption and prevents further AChR synthesis and clustering. Interestingly, the phenotype observed in $Agrn^{-/-}$ mice — the uniform, non-clustered distribution of AChRs — is partially rescued in mice that lack both agrin and ChAT[78]. Such feedback mechanisms may also help to explain why AChRs are only found at synapses: other proteins, such as MUSK and rapsyn, ensure AChR stability and thus counteract the effect of cholinergic neural activity.

Similar to AChR clustering, the accumulation of agrin in the extracellular matrix (ECM) must be controlled to maintain proper receptor clustering. This may be achieved by matrix metalloproteinases (MMPs), which regulate the ECM by cleaving matrix proteins[81]. For instance, purified MMP3 can directly cleave agrin, lowering its levels in the ECM[82–85]. MMP3-null mutant mice show an increased density of AChRs, as well as agrin immunoreactivity[82,85]. MMP3 is secreted in its pro-form and requires cleavage to become active (reviewed in REF. 86). This activation

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Table 1 | **Molecular signalling for NMJ maturation**

Molecules	Sources	Targets	Roles	Species	Refs
Postsynaptic maturation					
Agrin	NT, MF and SC*	MUSK–LRP4 complex	MUSK activation, leading to ↑AChR clustering	Mouse, frog and *D. melanogaster*	11,23,50, 54
LRP4	MF and NT[‡]	MUSK	MUSK activation, leading to ↑AChR clustering	Mouse	56–58,109, 110,112
WNT ligands	MF, NT and SC[§]	MUSK (WNT9A, WNT11, WNT16)	Agrin-dependent MUSK activation, leading to ↑AChR clustering	Mouse, zebrafish and *D. melanogaster*	61,64–66
		MUSK (WNT3)	Agrin-independent MUSK activation, leading to ↑AChR clustering		
		Target of WNT3A is not clear	Suppression of rapsyn expression, leading to ↓AChR clustering		
ACh	NT	AChRs	↓AChR synthesis and clustering	Mouse	78–80,185
Neuregulin 1	NT and SC[‖]	ERBB receptors	↑Synthesis and clustering of AChRs and associated proteins	Mouse	15,16,68, 71–74
TGFβ	SC	TGFBR1–TGFBR2 complex	↑Agrin expression	Frog	12
		Punt receptors	↑Agrin expression	*D. melanogaster*	13
Synaptic laminins	MF	Integrins on MF (for laminin β2), dystroglycan on MF (for laminins α4 and α5)	↑AChR clustering	Mouse	81,88,105
Presynaptic maturation					
Synaptic laminins	MF	N-type (early) or P/Q-type (late development) VGCCs on NT (for laminin β2); target for laminins α4 or α5 is not clear	• Regulation of active-zone proteins • Presynaptic organization	Mouse	6,88,89, 186
FGF7, FGF10 and FGF22	MF	FGFR2B on NT	↑Vesicle clustering	Mouse	93
Collagen α2, α3 and α6 chains (IV)	Not clear	Not clear	↑Vesicle clustering	Mouse	93
BDNF	MF	Pro-BDNF: p75NTR	Retraction of NT	Mouse	102,104
		Mature BDNF: TRKB	Nerve survival		
GDNF	MF and SC[¶]	Not clear	Regulation of number of innervating axons	Mouse	98,101,187
WNT ligands	MF, NT and SC[#]	Not clear	Presynaptic branching and maturation	*D. melanogaster*	113,114
LRP4	MF and NT	Not clear	• Presynaptic maturation (*in vivo*) • Synaptic vesicles and active-zone protein clustering (shown *in vitro*)	Mouse	110,112
TGFβ	SC	TGFBR1–TGFBR2 complex	Increase in nerve–muscle contacts and maturation	Frog and *D. melanogaster*	12,13
PSC maturation					
Neuregulin 1	NT and SC	ERBBs	SC proliferation and survival	Mouse	15–17
Synaptic laminins	MF	Not clear	Laminins β2 and α4 or α5 control SC proliferation and migration	Mouse	19,20
ATP	NT	P2YRs	SC activation	Mouse	18

ACh, acetylcholine; AChR, ACh receptor; BDNF, brain-derived neurotrophic factor; *D. melanogaster*, *Drosophila melanogaster*; FGF, fibroblast growth factor; FGFR2B, fibroblast growth factor receptor 2B; GDNF, glial-cell-derived neurotrophic factor; LRP4, low-density lipoprotein receptor-related protein 4; MF, muscle fibre; MUSK, muscle-specific tyrosine kinase receptor; NMJ, neuromuscular junction; NT, nerve terminal; P2YR, purinergic type 2Y receptor; p75NTR, p75 neurotrophin receptor; PSC, perisynaptic Schwann cell; SC, Schwann cell; TGFβ, transforming growth factor-β; TGFBR, transforming growth factor-β receptor; TRKB, tropomyosin-related kinase B; VGCC, voltage-gated calcium channel. *Neuronal isoform is more potent, muscle isoform is dispensable and SC isoform enhances AChR aggregation. [‡]Expression of neuronal LRP4 has not been determined (see main text for details). [§]At the *D. melanogaster* NMJ, SC-associated WNT (Wingless) signalling regulates glutamate receptor clustering. [‖]Not yet clear. [¶]Overexpression of GDNF by SCs did not cause hyperinnervation. [#]The SC-derived WNT-induced contribution to presynaptic regulation remains unknown.

might be activity-dependent, as denervated muscles exhibit high levels of agrin and presumably reduced levels of active MMP3 (REF. 85). Interestingly, MMP3 is found in the ECM around PSCs and is absent from active-zone areas[82,83,85]. Given the similar positioning of PSC processes and MMPs at the edge of the synaptic cleft, it is tempting to propose that MMPs that are from, or activated by, PSCs decrease perisynaptic levels of agrin, leaving it concentrated in the endplate area, where maximal AChR clustering is observed.

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Presynaptic maturation. Presynaptic nerve terminals also undergo major changes during NMJ formation. Specifically, there is an increase in the number of nerve ramifications and active zones, and in the levels of associated proteins; there are changes in the distribution and types of calcium channels expressed; and there is a decrease in the number of nerve terminals in contact with a single muscle fibre. Unlike postsynaptic maturation, fewer molecular signals that control presynaptic differentiation have been identified, and the molecular mechanisms that underlie presynaptic maturation are less well understood.

Laminins are glycoproteins that are considered to be important regulators of presynaptic terminal maturation. They are heterotrimeric (comprising α, β and γ subunits) muscle-derived glycoproteins and are included in the basal lamina of the ECM. The absence of laminins results in mild to severe defects in NMJ development[6,19,87,88]. For example, NMJs from mice that lack the laminin β2 chain (which is present in all synaptic laminins) have fewer active zones, synaptic vesicles that do not cluster and reduced synaptic activity. In addition, the processes of SCs in these animals invade the synaptic cleft[87,88]. Laminin β2 binds to presynaptic N-type voltage-gated calcium channels during early stages of development and to P-type and Q-type channels at later stages[81,89] (different channels are involved in transmitter release at these respective stages of development)[89,90]. The interaction of laminin β2 with calcium channels regulates active-zone proteins such as bassoon and the distribution of calcium channels[89,91]. Other synaptic laminins, such as laminin α4 and laminin α5, are required for NMJ maturation and for the proper alignment of pre- and postsynaptic elements (reviewed in REF. 81).

Certain fibroblast growth factors (FGFs) — namely, FGF7, FGF10 and FGF22 — promote NMJ maturation and vesicle clustering in motor neurons *in vitro*[92,93] and *in vivo* during embryonic and early postnatal development, as revealed using mice lacking FGF receptor type 2B (FGFR2B; *Fgfr2b*$^{-/-}$ mice)[93]. Muscle fibres are the main sources of FGFs, which signal at the NMJ mainly through presynaptic FGFR2B[93,94]. Another molecule involved in NMJ maturation is type IV collagen, which is found primarily in the basal lamina[93,95]. Vesicle clustering is promoted by collagen IV α2 chains during embryonic stages, and by collagen IV α3 and α6 chains during postnatal development[93]. Interestingly, FGFs (FGF7, FGF10 and FGF22), laminin β2 and collagen IV (α2, α3 and α6 chains) were all found to be important for presynaptic maturation, and the three classes of molecules were shown to act sequentially from embryonic to postnatal stages[93]. At least for FGF7, FGF10, FGF22 and collagen IV (α3 and α6 chains), this idea is consistent with the levels of expression of mRNA encoding these proteins at different developmental stages[93]. Type XIII collagen may also be important for pre- and postsynaptic maturation, as NMJs from mice that lack collagen XIII have fewer active zones, abnormal AChR clustering and improper presynaptic–postsynaptic alignment, all of which correlate with synaptic defects[96].

Synapse elimination
A reduction in the number of synaptic contacts that results from activity-dependent synaptic competition.

Finally, the extracellular domain of the signal regulatory protein-α (SIRPα), which is a member of the immunoglobulin family, promotes synaptic-vesicle clustering in cultured chick spinal cord motor neurons[97].

Glial-cell-derived neurotrophic factor (GDNF) and brain-derived neurotrophic factor (BDNF) also regulate presynaptic maturation. GDNF is expressed by muscle fibres and SCs, and promotes motor neuron survival[98,99]. Injection of GDNF, or the genetic overexpression of its muscle isoform, leads to an increase in the number of nerve terminals innervating the same NMJ and delays synapse elimination[100,101]. SCs also express GDNF, but the role of SC-derived GDNF in NMJ maturation remains unclear[101]. Like GDNF, the application of BDNF to the muscles of neonatal mice delays synapse elimination[102]. BDNF is initially synthesized as its precursor form (pro-BDNF), which is then cleaved to generate the mature form (mBDNF)[103]. Through different receptor interactions, pro-BDNF favours synapse elimination, whereas mBDNF promotes the maintenance of synapses[102,104]. Moreover, the conversion from pro-BDNF to mBDNF may be regulated by synaptic MMP3 and MMP9 (REFS 102,104). As MMP3 localizes in the ECM that surrounds PSCs[85], it is again appealing to propose that PSC-derived MMPs might convert pro-BDNF to mBDNF and thus influence presynaptic maturation.

Coordinated tri-directional maturation of the NMJ. In order to build synapses that are adapted and functionally tuned, the maturation of the NMJ must be well coordinated. Many molecules and pathways, from all three synaptic elements at the NMJ, are involved in aspects of this process (FIG. 1; TABLE 1).

Muscle-derived laminins that contain α4, α5 or β2 chains have an autocrine role in postsynaptic maturation[105]. Laminins containing the β2 chain bind to integrin receptors containing the β1 subunit in the ECM to increase AChR clustering, and laminins with an α4 or α5 chain bind to dystroglycan, which stabilizes AChRs in the postsynaptic membrane and participates in the recruitment of acetylcholinesterase to the NMJ[106–108] (reviewed in REF. 81). Thus, the anterograde and retrograde effects of synaptic laminins may be responsible for proper pre- and postsynaptic alignment. Laminins also control glial maturation: synaptic laminin 521 (which comprises α5, β2 and γ1 subunits) actively inhibits PSCs from extending their processes into the synaptic cleft[19]. In mice that lack the laminin β2 chain, the invasion of the NMJ synaptic cleft by PSC processes could explain many of the abnormalities in NMJ function, as PSCs could block synaptic transmission and neuron–muscle interactions[88]. Therefore, laminins coordinate the maturation of the NMJ and ensure the correct relative positioning of each synaptic element[19].

Like laminins, LRP4 acts bidirectionally to coordinate pre- and postsynaptic development. LRP4 acts as an agrin co-receptor to stimulate AChR clustering[57,58,109], but it is also necessary for presynaptic maturation (which is reflected by the clustering of synaptic vesicles and active-zone proteins)[110]. Muscle-derived LRP4 may act as a retrograde signal to regulate presynaptic maturation in a

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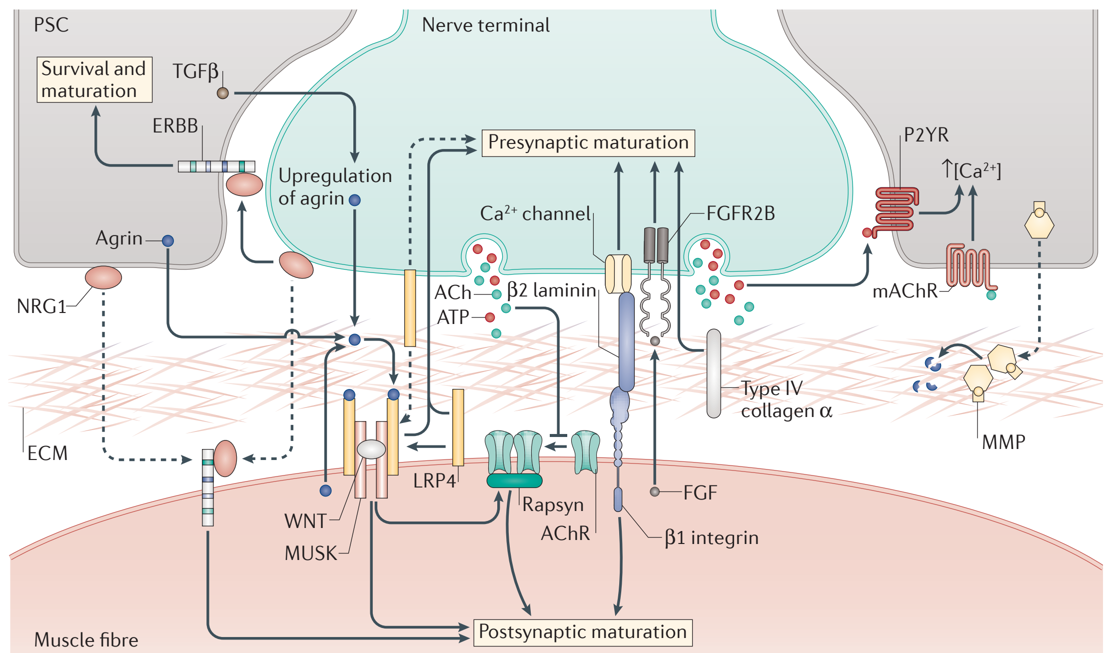

Figure 1 | **Synapse formation at the NMJ: generating a functional communication unit.** Parallel signalling pathways at the neuromuscular junction (NMJ) are involved in the coordinated maturation of the presynaptic terminal (green), the postsynaptic muscle fibre (red) and the perisynaptic Schwann cells (PSCs; grey); dotted lines indicate pathways that are still being debated. Agrin, which is released by the nerve terminal, muscle fibre and surrounding PSCs, acts on the LRP4–MUSK complex, which comprises low-density lipoprotein receptor-related protein 4 (LRP4) and muscle-specific tyrosine kinase receptor (MUSK). The phosphorylation of MUSK leads to rapsyn-mediated clustering of ionotropic acetylcholine receptors (AChRs) and postsynaptic maturation. AChR clustering can also be enhanced by WNT ligands (associated with MUSK in the figure), whereas release of ACh inhibits AChR clustering. LRP4 is mainly of postsynaptic origin, although neuronal LRP4 may also have a role (see main text for details). LRP4 acts as a co-receptor for agrin and stimulates AChR clustering as well as presynaptic maturation by clustering synaptic vesicles and active-zone proteins (not shown). Neuregulin 1 (NRG1) can be released by the nerve terminal and/or surrounding PSCs, and binds to PSC-expressed or postsynaptic ERBB receptors (ERBB2 or ERBB3). The binding of NRG1 to ERBB receptors on PSCs promotes PSC survival and maturation. Although the exact signalling pathway is controversial, the activation of postsynaptic ERBB receptors by NRG1 may increase levels of postsynaptic proteins such as rapsyn, MUSK and AChR, and could have a role in AChR clustering, which overall leads to postsynaptic maturation. Synaptic laminins, such as laminins β2, α4 and α5, are released by the muscle fibre. They form heterotrimeric glycoproteins that are included in the basal lamina and are important for proper pre- and postsynaptic alignment and maturation, as well as PSC maturation (not shown). Laminin β2 binds to presynaptic calcium channels and regulates active-zone proteins (not shown). Postsynaptically, laminins interact with integrin β1, which increases AChR clustering. Fibroblast growth factors (specifically FGF7, FGF10 and FGF22) are released by the muscle fibre and activate mainly presynaptic type 2B FGF receptors (FGFR2B); thus, they are important for vesicle clustering and presynaptic maturation, as are type IV collagen α chains (α2, α3 and α6). PSC-derived transforming growth factor-β (TGFβ) induces presynaptic maturation and postsynaptic differentiation by upregulating the expression of agrin. Synaptically released ATP is detected by PSC-expressed purinergic type 2Y receptors (P2YRs) and triggers increases in intra-PSC Ca^{2+} concentrations. PSCs also express muscarinic AChRs (mAChRs), and their activation by the local application of ACh triggers increases in intra-PSC Ca^{2+} concentrations. However, mAChRs are not activated by endogenous ACh release. Matrix metalloproteinases (MMPs) in the extracellular matrix (ECM) that surrounds PSCs regulate the composition of the ECM and cleave matrix proteins such as agrin, triggering its removal from the ECM.

MUSK-independent manner[110]. By contrast, MUSK may indirectly participate in presynaptic differentiation by clustering LRP4 (REFS 59,110,111), thus concentrating the bidirectional signalling of LRP4. Although the expression of LRP4 by muscle fibres has been established[56,57], a recent study suggests that neuronal LRP4 may also contribute to NMJ maturation[112]. More-severe NMJ deficits were observed in mutant mice that lacked both muscle- and motor-neuron-derived LRP4 than in mice that only lacked muscle-derived LRP4 (REF. 112). However, direct evidence of LRP4 protein or RNA expression specifically in motor neurons remains undetermined[112].

WNT ligands may also have effects beyond AChR clustering. For instance, disrupting Wingless protein activity in *D. melanogaster* results in both pre- and post-synaptic NMJ defects, which suggests that, at least in flies, Wingless proteins serve as both anterograde and retrograde signals to promote NMJ formation[113,114]. BDNF and GDNF could also have multiple roles at developing NMJs, as muscle fibres and SCs express receptors for both neurotrophic factors[115–117]. In neuron–muscle cultures, GDNF increases the membrane levels of AChRs in the muscle fibre without altering AChR synthesis[118], whereas in cultured myotubes BDNF can

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inhibit agrin-induced AChR clustering[115] and maintain AChR clusters[117]. Moreover, the regulation of PSC activity by neurotrophic factors at the adult mouse NMJ[119] indicates that such molecules may have broader effects on NMJ synaptogenesis.

As highlighted above, PSCs participate in many important steps of synapse maturation. SCs rely on pre- and postsynaptic elements for survival and maturation (BOX 1; TABLE 1), but they also contribute to pre- and postsynaptic maturation and NMJ maintenance. For instance, SCs are absent in mice that lack neuregulin 1, ERBB2 or ERBB3. Although contact formation seems to occur normally in these mice, nerve terminals subsequently retract and motor neurons degenerate shortly afterwards[16,72]. This strongly suggests that SCs are not important for the initial process of synapse formation but are essential for the survival of motor neurons and for the maintenance of NMJs during synapse maturation. SCs in general are important for neuronal survival[120,121], and PSCs may have similar roles at the NMJ. One study examined the contribution of PSCs at the NMJ using complement-mediated cell lysis to specifically ablate PSCs at the amphibian NMJ *in vivo*[10]. Without PSCs, the NMJs were smaller, had fewer branches and underwent a long-term loss of synapses[10]. Similar observations were made at the mouse NMJ following selective PSC ablation with a ganglioside-specific autoantibody[122,123].

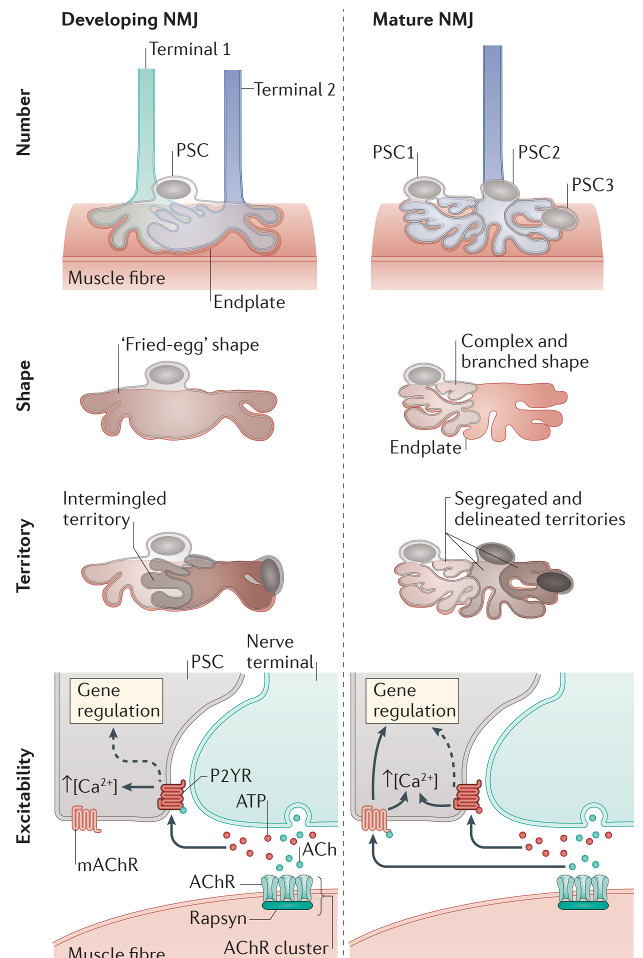
Interestingly, transforming growth factor-β1 (TGFβ1) has been proposed to mediate some of the synaptogenic effects of PSCs on the NMJ. TGFβ1 is expressed by PSCs, found in SC-conditioned media and promotes synaptogenesis in nerve–muscle co-cultures[12]. Indeed, in cultured spinal neurons treated with SC-conditioned media, TGFβ1 increased the size and number of AChR clusters at nerve–muscle contact sites, probably by upregulating agrin expression[12]. *In vivo*, glial-cell-derived TGFβ was also shown to be a synaptogenic factor and modulated synapse development at the *D. melanogaster* NMJ. Glial TGFβ activates muscular TGFβ receptors and thus increases retrograde muscle signalling and facilitates synaptic growth[13]. Moreover, recent data from our laboratory suggest that PSCs may

Ganglioside
Member of a family of oligoglycosylceramide plasma-membrane lipids that was originally discovered after its isolation from ganglion cells and which is predominantly found in the nervous system. Antibodies against some disialosyl epitopes of gangliosides can be used to specifically ablate perisynaptic Schwann cells at the mammalian neuromuscular junction.

Box 1 | **Maturation of PSCs**

Perisynaptic Schwann cells (PSCs) are generated from stem cells that originate from the neural crest and follow motor axons into the muscles, where they reach their final location at the neuromuscular junction (NMJ) (reviewed in REFS 5,162). Immature Schwann cells begin to take on a PSC phenotype once they reach their location at the NMJ. As the number of PSCs at an NMJ increases[18,150,151], their shapes become increasingly complex, their motility decreases and their territories become segregated (see the panels in the figure illustrating the changes in the number[18,149,151], shape[149] and territory[149] of PSCs). These changes have been attributed to the spatial constraints that dictate PSC territories, suggesting that PSCs actively compete for available space[149]. Interestingly, this period of PSC remodelling coincides with the peak of postnatal maturation of the NMJ; in mice, this occurs during the first postnatal week. During this period, PSCs show a high level of phagocytic activity[146] when pruning connections during synapse maturation[21,22], and they express receptors to detect and decode neurotransmission[18].

Interestingly, the PSC receptors that detect neurotransmission differ between developing and mature NMJs (see the figure panel labelled 'excitability')[18,154]. Whereas PSCs at immature NMJs rely mainly on purinergic signalling[18], PSCs at mature NMJs use purinergic receptors (including purinergic type 2Y receptors (P2YRs)) and muscarinic metabotropic acetylcholine receptors (mAChRs) to detect released neurotransmitters[154]. This differentiation may be important for regulating the expression of a number of genes, particularly those under the control of mAChR activation[163], such as the gene encoding glial fibrillary acidic protein (GFAP). One intriguing possibility is that the receptor types involved may be adapted to the state of NMJ plasticity, such that low mAChR activity is permissive for growth and remodelling, whereas higher mAChR activation promotes stability and the modulation of synaptic activity and efficacy.



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Retraction bulb
Enlarged distal part of the axon undergoing retraction (that is, axosomal shedding); it is commonly observed during synapse elimination.

either permit or restrict NMJ expansion and that these effects depend on the activation of PSCs by released neurotransmitters (ACh or ATP) and on the maturity of the synapse[18] (FIG. 1).

Activity-dependent NMJ maturation

Although the NMJ is functional upon its formation, it undergoes maturation for a prolonged period. Like all synapses in the nervous system, it depends on synaptic activity to develop an optimal organization and proper synaptic efficacy to accomplish its function. Activity shapes both the postsynaptic apparatus[8,78,79] and presynaptic organization, as well as synaptic function. One of the most fascinating activity-induced changes at the NMJ is the dramatic decrease in the number of synaptic connections. In mature NMJs, a single nerve terminal contacts the postsynaptic cell; however, during early stages of development, each muscle fibre is innervated by several nerve terminals that branch from different motor neurons[44,45]. As a result, competition ensues between nerve terminals for the same postsynaptic endplate area. This competition is activity-dependent and leads to the elimination of supernumerary inputs; it is regulated by pre- and postsynaptic elements and by PSCs associated with the synapse. A similar activity-dependent competition occurs between regenerated axons during the reinnervation of muscle fibres following nerve injury (BOX 2).

Unlike the pathways that regulate pre- and postsynaptic maturation, little is known about the molecular mechanisms that regulate activity-dependent synaptic competition. Moreover, the relationship between the activity-dependent processes and the molecular pathways that drive synapse formation and maturation remains ill defined. We discuss below synaptic competition and elimination, with a particular focus on the possible underlying molecular mechanisms and the role of glial cells during these processes.

Synaptic competition and elimination. Between the late embryonic period and the early postnatal period, nerve terminals at the same NMJ compete with one another for the sole innervation of the muscle fibre[4,45,124,125]. This process is important for the development of proper synaptic connections and synaptic function[43,44,126,127]. *In vivo* time-lapse imaging of synaptic elimination at dually innervated NMJs revealed that one axon progressively vacates a synaptic area, which is then gradually taken over by a competing nerve input[125,128]. Typically, a 'losing' terminal ultimately retracts in the form of a 'retraction bulb', which is eliminated by PSCs[21,125]. This competition process is highly dynamic, and competing terminals continuously vie for territories. Indeed, the outcome of the competition is not preordained, and the territories of nerve terminals can even shift back and forth[125], such that a terminal that initially occupies a smaller area could take over the territory and ultimately 'win' the competition[125]. Interestingly, a recent study showed that terminals that were considered to be losing the competition (that is, smaller terminals) — and even retraction bulbs at a distance from the endplate — were able to reverse their fate and grow back to occupy the NMJ if their competitor was ablated[129].

The difficulty in predicting a winning nerve terminal solely on the basis of morphological and structural observations suggests the mechanisms involved in synaptic competition and elimination are complex. It has been suggested that the outcome of synaptic competition and elimination depends on the efficacy and structure of nerve terminals — properties that are dependent on neuronal activity[130–132]. The general idea is that, at early stages of synaptic competition (for example, during the first postnatal days in mice), terminals that compete at the same NMJ could have comparable synaptic efficacies and presynaptic areas that occupy equally sized postsynaptic territories. Then, one input is gradually strengthened and occupies a larger area at the expense

Box 2 | NMJ formation following injury and in disease

The peripheral nervous system has a remarkable ability to regenerate[164,165]. Following nerve damage, the distal sections of injured axons remain functional for a given period before Wallerian degeneration occurs. This time period is known to directly depend on the length of the degenerating nerve stump[166,167]. Wallerian degeneration involves many events: changes to the blood–nerve barrier, such as increased permeability; the degeneration of the distal axon; the dedifferentiation and proliferation of Schwann cells, which start invading the synaptic cleft[168,169]; the recruitment of macrophages; and the reorganization of the extracellular matrix[170]. The combined goal of all of these events is to generate an innervation-permissive environment so that regenerated axons can reinnervate the muscle endplate once Wallerian degeneration is complete.

During reinnervation, axons continue to grow beyond the boundary of a denervated neuromuscular junction (NMJ; which is delineated by the endplate area and the clustered acetylcholine receptors (AChRs)), where they travel along peripheral Schwann cell bridges that have been formed during denervation[165]. These 'escaped fibres' can reach another synaptic site, leading — along with the axonal reinnervation — to the polyinnervation of NMJs[171,172].

Remarkably, reinnervation shares several features with synapse formation during development. First, in both cases, NMJs that are polyinnervated undergo a period of synaptic competition, which leads to monoinnervation[171–173]. Second, as with elimination in the developing NMJ, elimination during reinnervation depends on and is shaped by synaptic activity[137,174]. Therefore, there is evidence to suggest that the processes that occur during NMJ formation are recapitulated following nerve injury. This would imply that during reinnervation perisynaptic Schwann cells (PSCs) might adapt their sensibility in response to neurotransmission until it resembles that observed in immature PSCs. This suggests that the relative contributions of muscarinic and purinergic signalling in PSCs can also be adapted.

Although these NMJ-forming processes can be repeated, severe diseases of the NMJ can arise if a single step of NMJ formation is compromised. For example, the production of autoantibodies targeting specific proteins that are present in the endplate leads to neuromuscular diseases such as myasthenia gravis, which is characterized by muscle weakness and fatigue. Autoantibodies that are involved in the more prevalent neuromuscular diseases include those that target AChRs[175], muscle-specific tyrosine kinase receptor (MUSK)[111,176–178] and low-density lipoprotein receptor-related protein 4 (LRP4)[179,180] (reviewed in REF. 181). Furthermore, mutations in the genes encoding proteins that are essential for NMJ formation, such as MUSK[59] or proteins associated with the basal lamina, lead to congenital neuromuscular diseases[182]. Recently, motor-endplate disease, a severe genetic disorder in mice that is characterized by progressive muscular weakness and that leads to paralysis and premature death, has been attributed to a mutation in the gene encoding the voltage-gated sodium channel Nav1.6. Interestingly, this mutation leads to the apoptosis of PSCs during synapse formation[183,184].

Thus, a better understanding of NMJ formation and maturation will help to elucidate both the regenerative processes that occur after nerve injury and how altered maturation leads to major defects. Moreover, considering the NMJ as a functional unit composed of a presynaptic terminal, a muscle fibre and associated PSCs may lead to the identification of additional therapeutic targets to treat neuromuscular diseases.

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of the other nerve terminals[44,133]. At dually innervated NMJs, a disparity in the synaptic efficacies of competing inputs gradually builds up, resulting in a clear distinction between a strong input (with a high release of neurotransmitter) and a weak input (with a low release of neurotransmitter)[18,130,132] (FIG. 2).

Although initially a controversial idea[134–136], there is evidence to suggest that synaptic inputs that are more active are favoured during synaptic competition[131,137–141]. For instance, Buffelli *et al.*[131] deleted ChAT in a subset of motor axons in mice to cause a robust neuronal-subset-specific reduction in neurotransmission and hence a disparity in synaptic efficacy between affected and unaffected subsets. This manipulation resulted in maintenance of the more-active inputs and elimination of the others[131]. In addition to relative synaptic efficacy, the pattern of activity of competing terminals (spike timing) is an important determinant of synapse elimination. During the early phase of synaptic competition (postnatal day 0 (P0) to P4), motor-unit activity from competing terminals is synchronous, but these inputs become gradually asynchronous during the period when synapse elimination is prominent (P4–P10). Motor units reach complete asynchrony at the end of the synaptic competition and elimination process (at ~P14 in mice)[139,142]. Interestingly, inducing asynchrony between spikes from competing axons promotes synapse elimination, whereas imposed synchrony extends the period of synaptic competition[137].

Asynchrony — that is, distinct firing patterns from different presynaptic terminals — could allow the muscle fibre and/or PSCs to detect and distinguish between independent inputs and then actively participate in the strengthening or weakening of individual terminals. For example, focal blockade of AChRs in a portion of an endplate area caused presynaptic retraction from this specific area, whereas blockade of an entire endplate area did not induce presynaptic loss[138]. This suggests that the postsynaptic element must distinguish between

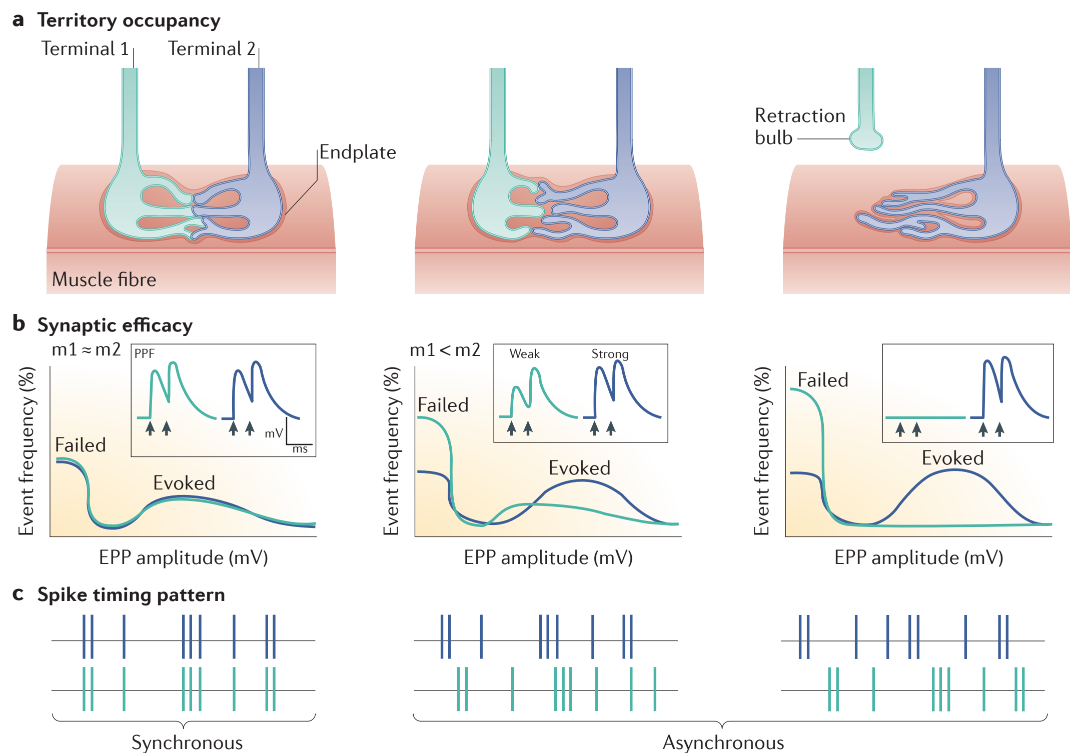

Figure 2 | **Synaptic competition and elimination at the NMJ.** Morphological and functional changes during postnatal maturation are associated with synaptic competition and elimination. **a** | A dually innervated neuromuscular junction (NMJ) with two nerve terminals (green and blue) competing for the same endplate (represented by the darker red on the pale red muscle fibre) is represented schematically. Competing terminals start with similar territories (left panel; territories could be intermingled, but this is not shown here). Gradually, territories segregate and one terminal (blue) occupies a larger area (middle panel), leading to the elimination of the losing axon (right panel). **b** | Competing terminals could have a similar synaptic efficacy (left panel), as defined by the small differences in quantal content 'm', the similar rate of failed and evoked endplate potentials (EPPs), and the similar paired pulse facilitation (PPF) profiles of each of the terminals (left graph). One input (blue) gradually becomes stronger (middle graph), with a larger quantal content, lower failure rate and lower PPF (which is defined as the increase in the amplitude of the second EPP compared to the first one) and a larger number of evoked EPPs with higher amplitudes. (Experiments supporting these observations were performed in a condition of low probability of release, with low Ca^{2+} and high Mg^{2+} concentrations)[18,130,132]. Once the losing terminal (green) retracts (right graph), it no longer induces synaptic activity at this given NMJ (100% failure). **c** | Patterns of neuronal firing change from synchronous motor-unit activity (left panel) to asynchronous activity (middle and right panels), which leads to synapse elimination. The functional changes depicted in parts **b** and **c** are indirectly associated with the morphological changes presented in part **a**.

Spike timing
Pattern of temporal correlation between presynaptic and postsynaptic activities. Synchronous and asynchronous patterns of activity are observed during early and late phases of synapse elimination, respectively.

Asynchronous activity
Uncorrelated timing of synaptic inputs onto the muscle fibre, leading to an out-of-phase activation.

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inputs to promote survival or elimination. More-active nerve terminals are also more likely to fire out of synchrony with their 'quieter' competitors; therefore, a number of spikes will occur in the most active input when its competitors are silent[137]. Thus, both the level and the pattern of activity shape synaptic competition and elimination.

Although a direct link between synaptic strength, structure and elimination has yet to be elucidated, there is evidence to support the notion that these three factors are interdependent[44,129,131]. For example, a slight decrease in synaptic activity could weaken the interaction between a terminal and a muscle fibre, resulting in a vacated territory as the less active terminal is remodelled. This territory could then be occupied by the competing terminal, and this new occupation may increase the feedback from the muscle fibre and PSCs to reinforce this terminal and result in improved efficacy. An experiment in which both synaptic activity and the territory occupancy of competing terminals could be monitored and modulated would provide valuable information on the relationship between synaptic organization and activity during synapse competition. In parallel with the effects of synaptic activity and territory occupancy, competing terminals may constantly battle against each other for survival factors such as GDNF and BDNF[101,102,104]. Indeed, pro-BDNF and mBDNF serve as punishment and reward signals, respectively, to either eliminate or maintain competing nerve terminals, leading to the maintenance of more-active inputs[102,104]. Thus, this would ultimately favour a nerve terminal that has a larger synaptic strength and a better pattern of synaptic activity, and that is in a more advantageous position to benefit from survival factors.

The process of synaptic competition requires a constant remodelling of the NMJ such that the molecular mechanisms that are involved in synapse maturation may also be dynamically regulated during synaptic competition. The elimination of nerve terminals must involve activity-dependent molecular mechanisms to weaken some nerve–muscle interactions while stabilizing others. Hence, one could hypothesize that the mechanisms involved in AChR clustering and stabilization (including MUSK signalling and receptor anchoring by rapsyn) are regulated in an activity-dependent manner. As some terminals are eliminated, the postsynaptic territory can be either re-occupied by neighbouring inputs or left vacated[22,125]. If left unoccupied, the postsynaptic apparatus will be remodelled: the improperly localized AChRs will be dispersed and removed[125,143]. Consistent with this possibility, during postnatal development the endplate area changes from an 'en plaque' oval structure to a branched 'pretzel-like' shape owing to the loss of AChRs from some regions[143]. As ACh regulates the expression and clustering of AChRs, this transition could be activity-dependent[77–80]. Indeed, this physical transformation temporally corresponds with synaptic competition and elimination events, which themselves are activity-dependent[8,143]. Moreover, activity-dependent changes may be regulated by many molecules, such as laminins, as the transition into a pretzel-like shape is delayed at NMJs in mice that lack laminin α5 (REF. 105).

Glial roles in synaptic competition and elimination. As axons gradually retract, they leave behind debris-containing synaptic organelles known as axosomes[21,144] (FIG. 3). These are formed by the engulfment of axonal endings by PSCs and are subsequently entirely contained within these cells[21]. Axosomes have been observed following the elimination of terminal branches — leading to segregation of the territories of competing terminals[21] — and during the retraction of nerves as retraction bulbs, which leads to synapse elimination[21] (FIG. 3a). Consistent with the well-known role of glial cells in debris clearance[145], the retraction of terminals is associated with high lysosomal activity, which suggests that axosomes are processed through the lysosomal pathway of SCs[22,146]. Interestingly, axonal debris was cleared more slowly in a mouse model of a lysosomal storage disease[146]. At the fly NMJ, the phagocytosis of presynaptic debris results from coordinated efforts of glial and muscle cells[147,148]. These cells phagocytose presynaptic debris, including debris from weak and unstable synaptic boutons. This is an activity-dependent phenomenon, as the accumulation of debris increases following high-frequency stimulation of motor neurons[148].

With regard to synapse reorganization and elimination, a key question is how the nerve terminals to be maintained — or pruned and eliminated — are identified. Two recent studies implicate PSCs in synapse elimination. First, Smith *et al.*[22] showed that during development, PSCs arrive at the NMJ and cover all terminals with no apparent preference. Early in the process of synapse elimination (that is, during embryonic and early postnatal stages), PSCs extend their processes between the nerve terminals and muscle fibres, obstructing AChRs and synaptic communication. It has been proposed that PSCs compete with nerve terminals to be in close proximity to AChR-covered surfaces in order to make even more contact with AChRs than the terminal boutons. PSCs then phagocytose and remove synaptic branches from different terminals, resulting in the random elimination of terminals. The eliminated terminals leave behind vacant territory that can be occupied by non-eliminated neighbouring inputs[22] (FIG. 3b). Interestingly, given the tight relationship between AChRs and glial coverage, it has been proposed that PSCs may also sculpt the postsynaptic endplate[22], perhaps via an increase in MMP3 activity, which would cause the cleavage of agrin and thus the elimination of unstable AChRs[83,85]. Such mechanisms could contribute to the transition of the endplate into a pretzel-like shape, and would be consistent with the change of PSCs from a 'fried-egg' shape to a branched morphology during postnatal development[149] (BOX 1). However, these hypotheses remain to be tested.

Smith *et al.*[22] propose that PSCs do not select a winning nerve terminal per se but instead randomly eliminate branches, eventually leading to a sole survivor. This study provides extensive and important information on the ultrastructural organization of polyinnervated NMJs. It also highlights the complex structural interaction between competing nerve terminals, the postsynaptic apparatus and the surrounding PSCs. However, it does not fully explain the role of neuronal activity in

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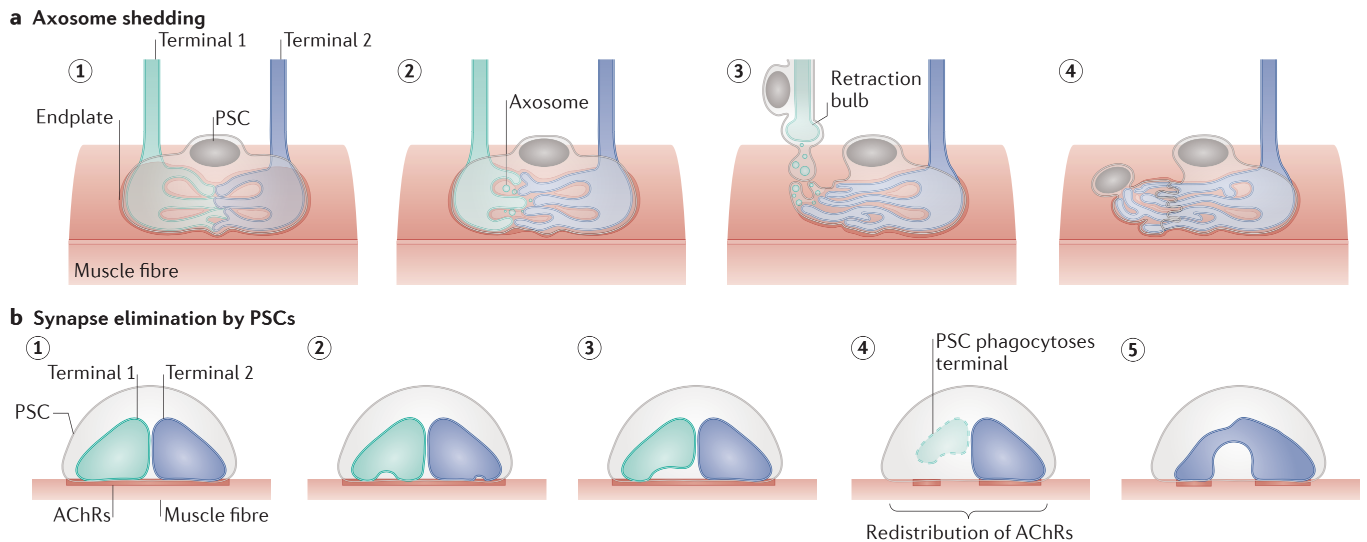

Figure 3 | **PSCs eliminate supernumerary connections.** **a** | A schematic representation of the losing nerve terminal shedding axosomes[21,144] is shown. Step 1: during synaptic competition, competing nerve terminals (in green and blue) may occupy similar territories of the muscle fibre endplate (red) at the neuromuscular junction and are covered by a perisynaptic Schwann cell (PSC; translucent grey). Step 2: PSCs are thought to engulf the endings of nerve terminals, leaving organelles containing synaptic debris (axosomes) that are processed by the PSC lysosomal pathway (not shown). This decreases the size of the territory occupied by the losing terminal (terminal 1; green). This territory can be gradually occupied by its competitor (terminal 2; blue). Step 3: PSCs continue to phagocytose the losing terminal until the nerve retracts into a retraction bulb, which is ensheathed by Schwann cell processes. Step 4: following the elimination of the losing terminal, a single terminal (terminal 2; blue) innervates the muscle fibre. **b** | Mechanism of synapse elimination by PSCs[22] is shown. Step 1: a schematic representation shows a PSC (translucent grey) covering two nerve terminals (terminal 1, green; terminal 2, blue), which are competing to innervate the same muscle fibre (red). Steps 2 and 3: the PSC seems to compete with nerve terminals for acetylcholine receptors (AChRs; red band) by extending its processes between the nerve terminals and the muscle fibre, partially blocking synaptic neurotransmission. Step 4: the distribution of AChRs can be remodelled in places that no longer receive neurotransmitters. In parallel, the PSC phagocytoses nerve endings (in this case, the green terminal) that are in contact with the muscle fibre, on a stochastic basis[22]. Step 5: the vacated area can be occupied by a nearby terminal (blue).

this scenario. Our group proposes another mechanism by which PSCs might be involved in synapse competition and elimination[18]. As weaker terminals are more likely to be eliminated, PSCs may be able to differentiate between weaker and stronger terminals, and could therefore actively participate in the targeted elimination of 'losing' synapses. PSCs must possess certain qualities to actively decide which synapses to eliminate. As exemplified by the fact that most NMJs on the soleus muscles are covered by a single PSC during this period[18,150,151], a single PSC must be able to discriminate between competing inputs and accurately compare their relative synaptic strengths (as relative synaptic strength is a major determinant of synaptic competition). This condition was tested at dually innervated NMJs using a nerve–muscle preparation in which the activity of individual terminals could be independently evoked and recorded while observing the increases in intra-PSC Ca^{2+} levels (which reflect PSC activity) that were evoked by synaptic activity[152–154]. We showed that a single PSC at a dually innervated NMJ detects neurotransmitter that is released from each nerve terminal[18] (FIG. 4). Moreover, Ca^{2+} responses that were induced within the same PSC by neurotransmission from different competing terminals were proportional to the relative synaptic efficacy of each terminal (FIG. 4).

This tight relationship between terminal synaptic efficacy and intra-PSC Ca^{2+} responses does not only correspond to the amounts of neurotransmitter released, but also depends on intrinsic PSC properties, including PSC-receptor segregation[18]. Indeed, a single PSC seems to organize its receptors in functional groups to face each competing terminal[18], an arrangement that could enable a more efficient detection of released neurotransmitter and a more accurate differentiation of competing inputs. When more than one PSC is present at a dually innervated NMJ, each PSC differentiates the weak input from the strong input using similar mechanisms[18]. Thus, PSCs integrate synaptic properties by constantly monitoring the strength of competing terminals. This also implies that PSCs must provide specific feedback that either promotes or represses synapse elimination. Although such a feedback mechanism has not yet been elucidated, PSCs could target elimination machinery specifically towards weaker inputs, or — as PSCs can regulate synaptic activity at mature NMJs (see below)[153,155] — they could differentially modulate the efficacy of competing terminals, preferentially strengthening one terminal over the other.

The mechanisms proposed by Smith *et al.*[22] and by our group[18] are not mutually exclusive and could be complementary during synaptic competition. Indeed, at late embryonic and early postnatal stages, many motor

PSC-receptor segregation
Spatial grouping and separation of receptors on the surface of perisynaptic Schwann cell (PSC) processes. Presumably, this grouping enables the PSC to detect neurotransmitter release from each competing terminal at dually innervated neuromuscular junctions. This segregation was described for purinergic type 2Y receptors, which mediate intra-PSC calcium activity during synaptic competition.

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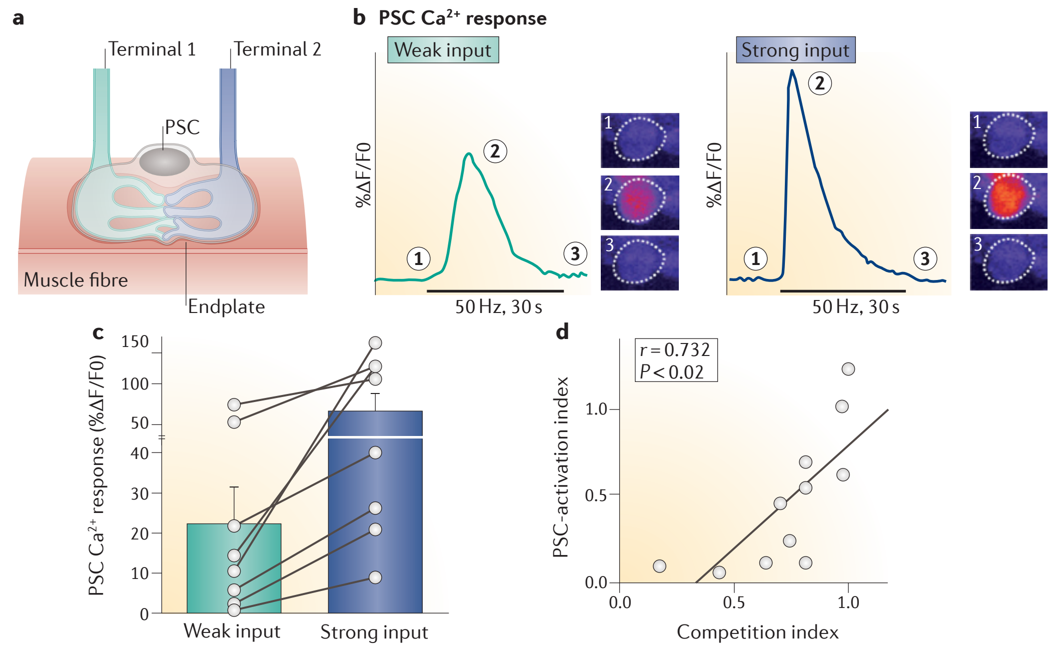

Figure 4 | **PSCs can differentiate between competing synaptic terminals.** **a** | A single perisynaptic Schwann cell (PSC; translucent grey) in close association with two terminals (green and blue) during synaptic competition is shown. **b,c** | PSCs can distinguish between strong (blue) and weak (green) inputs. Neurotransmitter release evoked by selective stimulation of the motor neuron associated with the stronger input induces intra-PSC Ca^{2+} responses of a greater amplitude (as indicated by the larger percentage change in the calcium-indicator fluorescence over basal fluorescence; %ΔF/F0) than those induced by neurotransmitter release from the weaker input. Insets in part **b** are false-colour confocal images of changes in intra-PSC Ca^{2+} level before stimulation (inset 1), at the peak of the response (inset 2) and after stimulation (inset 3). Part **c** shows intra-PSC Ca^{2+} responses (mean ± SEM) that were induced by strong and weak competing terminals. Each point represents a single experiment, and the black bars join the intra-PSC Ca^{2+} responses from the weak and strong nerve terminals that are competing at the same neuromuscular junction (NMJ). **d** | The amplitudes of intra-PSC Ca^{2+} responses (the PSC-activation index, which is defined as the amplitude of the intra-PSC Ca^{2+} response induced by the weak input divided by the amplitude of the intra-PSC Ca^{2+} response induced by the strong input) are tightly correlated with the synaptic strength (the competition index, which is defined as the quantal content of the weak input divided by the quantal content of the strong input and is a major determinant of the outcome of synaptic competition) of competing terminals. Each point is a single experiment, and the black line represents the linear regression relationship (*r*) between the PSC-activation and synaptic competition indices. Terminals with higher competition and PSC-activation indices are less likely to be eliminated. Parts **b–d** have been modified and republished with permission of the Society for Neuroscience, from: Glial cells decipher synaptic competition at the mammalian neuromuscular junction. Darabid, H., Arbour, D. & Robitaille, R. *J. Neurosci.* **33**, 1297–1313 (2014); permission conveyed through Copyright Clearance Center, Inc.[18].

neurons innervate the same NMJ (muscle fibres could typically receive more than ten different inputs)[43], and this polyinnervated stage coincides with the arrival of the first PSCs. Regardless of their number, PSCs are in contact with all terminals and show no apparent preference[22,43]. In this situation, PSCs may randomly eliminate branches from different terminals, as suggested by Smith *et al.*[22]. Gradually, the number of competing inputs would decrease and the segregation of territories would increase, such that each competing terminal would occupy a precise and delineated territory. When only a few competing terminals remain at an NMJ, PSCs — which can decode the relative synaptic efficacy of different inputs — could trigger the elimination of the weaker inputs. In doing so, PSCs may ensure that the most efficient and suitable nerve terminal is maintained.

Once mono-innervation is achieved, presynaptic and postsynaptic elements continue to rely on PSCs for proper functioning. Indeed, PSCs modulate the fine-tuning of neurotransmission at mature frog and mouse NMJs[153,155] in a Ca^{2+}-dependent manner[153,155], whereby the outcome of synaptic regulation is governed by the amplitude and kinetics of intra-PSC Ca^{2+} signalling. The ablation of PSCs at the mature amphibian NMJ results in structural and functional deficiencies[10], including an increase in nerve-terminal retractions, and decreases in both nerve-evoked muscle-twitch tension and the presynaptic release of neurotransmitter[10]. Thus, PSCs are important for synapse maintenance and refinement, as well as for synapse maturation and activity-dependent changes.

Conclusions and perspectives

Owing to its simplicity and accessibility, the NMJ has contributed substantially to our knowledge of synapse formation and maturation. These processes rely on complex molecular interactions that involve synaptogenic factors from muscle fibres, nerve terminals and PSCs. These factors ensure a coordinated maturation

Muscle-twitch tension
Tension elicited by a muscle contraction that is evoked by a suprathreshold stimulation of the muscle or the nerve input.

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of the NMJ such that presynaptic, postsynaptic and glial partners are morphologically matched and functionally tuned. This coordination is essential to accurately position the hundreds of presynaptic active zones to face the complex postsynaptic organization at the motor endplate. This tight presynaptic–postsynaptic pairing is further compounded by PSC processes, which express receptors that allow them to regulate NMJ efficacy[153,155].

The maturation of synapses at the NMJ is influenced by changes in synaptic activity and can therefore be tuned as a function of the environment. Moreover, such activity shapes NMJ connectivity by promoting the maintenance of certain inputs and the elimination of redundant connections after competition.

Recent studies have revealed that PSCs regulate many aspects of NMJ formation — from receptor clustering and presynaptic differentiation to activity-dependent synapse pruning and the modulation of synaptic activity. Interestingly, although synapse formation is best studied at the NMJ, the contribution of PSCs to synaptogenesis is less well understood than that of their CNS counterparts[156,157]. Indeed, many CNS glial-dependent pathways have been described, including those involving glypicans, thrombospondins, hevin and SPARC (secreted protein acidic and rich in cysteine)[158–161]. Similar mechanisms are probably involved at the NMJ; however, an assessment of these mechanisms is complicated by the lack of genetic and molecular tools for specifically targeting PSCs. Therefore, it will be important to develop such tools to unveil the contributions of PSCs and to better understand NMJ formation and maturation.

Furthermore, a functional link between the activity-dependent processes discussed here and the molecular pathways that drive synapse formation and maturation is yet to be elucidated. Considering the high number of intricate interactions among the three elements, it is possible that the molecular mechanisms involved are under some form of activity-dependent regulation. Furthermore, if this is the case, then the activity-dependent regulation of the molecular pathways of each compartment must be accurately controlled to shape a tightly organized communication unit. In summary, determining how the different molecular and activity-dependent mechanisms influence one another is an important issue that needs to be addressed.

Understanding NMJ formation and the contribution of glia to this process would have a marked impact on our knowledge of the changes that occur at the NMJ with ageing, following nerve injury or in the contexts of various motor neuron diseases. In particular, understanding the mechanisms that regulate the sprouting and extension of PSC processes would prove to be invaluable, as these are some of the most important events that regulate NMJ reformation following injury. Studying NMJ formation as a tripartite, integrated ensemble process would therefore give us a better understanding of numerous diseases, thus potentially expanding the spectrum of therapeutic targets.

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Competing interests statement
The authors declare no competing interests.

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