INDUCTION, ASSEMBLY, MATURATION AND MAINTENANCE OF A POSTSYNAPTIC APPARATUS

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The postsynaptic apparatus of the skeletal neuromuscular junction, like that of other synapses, contains a high-density patch of neurotransmitter receptors that is closely associated with a variety of extracellular, transmembrane and cytoplasmic proteins that have adhesive, structural and signalling roles. The postsynaptic apparatus is organized by signals from the presynaptic nerve terminal. It changes in shape, size and molecular architecture as it matures. Once mature, it can be maintained for the life of the organism, but has the capacity for remodelling in response to altered input. The molecular and cellular mechanisms that govern each of these stages are now being elucidated by a combination of microscopic and genetic methods, allowing the neuromuscular junction to serve as a model for smaller and less-accessible central synapses.

DEVELOPMENT

Although many of the main steps in neural development were described by the middle of the last century, our understanding of their mechanistic bases has remained rudimentary until recently. During the past decade, we have learned a few things about many critical developmental phenomena, and a lot about a few very restricted topics in this field[1]. The formation of the postsynaptic membrane at the vertebrate skeletal neuromuscular junction (NMJ) falls into the latter category. At this synapse, motor nerve terminals seem to organize postsynaptic differentiation by releasing a proteoglycan called agrin. Agrin activates a receptor tyrosine kinase called muscle-specific kinase (MuSK) on the myotube surface, which leads to clustering of acetylcholine receptors (AChRs) and other postsynaptic components through association with a cytoplasmic linker protein called rapsyn. Our first aim in this review is to argue that these interactions provide the core of a satisfying, albeit incomplete, understanding of a main step in synaptogenesis. Our second aim is in some ways the opposite: to point out numerous aspects of postsynaptic differentiation for which there is no molecular explanation as yet. The good news is that recently devised microscopic and genetic tools are now allowing descriptions of these steps *in vivo* at sufficient resolution to facilitate the quest for their underlying mechanisms.

Here we consider only the NMJ, but believe that the developmental mechanisms identified in this system are relevant to other chemical synapses[2], and might therefore be of general interest to neuroscientists. Assembly of the postsynaptic apparatus (the neurotransmitter receptors and their associated signalling components) at central synapses is a crucial step in the formation of neural circuits. Activity-dependent modifications of this apparatus, which are thought to underlie some forms of learning, make use of developmental mechanisms. Although the NMJ does not process a great deal of information, it does develop just as well as any other synapse, and it is maintained in a dynamic equilibrium that can be modified by activity. It is, therefore, not a bad model synapse. But what makes it a good one?

Why the NMJ and its postsynaptic membrane?

The three most widely appreciated experimental advantages of the NMJ are its large size (FIG. 1), its relative simplicity and its unparalleled accessibility[3]. Owing to these features, the concept of the 'postsynaptic

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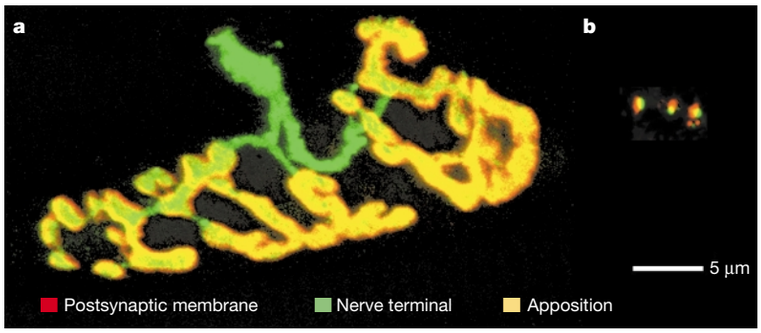

Figure 1 | **The neuromuscular junction. a** | The neuromuscular junction of an adult mouse. **b** | Three synapses on cultured mouse hippocampal neurons shown at the same scale.

receptor' was born at the NMJ a century ago[4]. Since then, these features have not only facilitated structural, functional and molecular analyses, but have also allowed their correlation with each other.

Accessibility comes at a price, though; NMJs are easy to see, partly because they are well separated from each other. Consequently, the specific activity of synaptic components (moles per gram of tissue) and the abundance of their messenger RNAs in muscle are low. Luckily, nature has provided a way to circumvent this limitation. The electric organ of *Torpedo californica* is essentially a hypertrophied NMJ from which the contractile components have been lost[5], so the specific activity of AChRs in the electric organ is three orders of magnitude higher than in muscle[6]. Indeed, most of the essential postsynaptic components that we discuss below, including agrin, MuSK, rapsyn, acetylcholinesterase, dystrobrevin and the AChRs themselves, not to mention many synaptic vesicle proteins, were initially isolated from *Torpedo* electric organs[5].

A deadly snake — *Bungaris multicinctus* — provided the fourth advantage in the study of the NMJ. A main component of its venom is a 74-amino-acid polypeptide — α-bungarotoxin (BTX) — which binds specifically and quasi-irreversibly to muscle and electric organ AChRs[7]. ^{125}I-BTX was initially used to identify AChRs, agarose-BTX is used to purify AChRs, and rhodamine-BTX is used to localize AChRs and to track their movements. The importance of BTX cannot be overstated. If a similarly useful ligand for a key presynaptic component had been available, our knowledge of postsynaptic development might not have outstripped so greatly our understanding of presynaptic development.

The phenomenon

Once myoblasts fuse to form myotubes, they begin to transcribe AChR subunit genes (α, β, γ and δ), translate the subunits, assemble functional pentamers ($\alpha_2\beta\gamma\delta$), and insert them into the membrane at a density that reaches ~1000 μm^{-2}. In adult muscle, by contrast, the density of AChRs is >10,000 μm^{-2} directly beneath the motor nerve terminal, but falls within several micrometres to <10 μm^{-2} in the extrasynaptic membrane[8] (FIG. 2a,b).

Muscles use at least four distinct mechanisms to effect this transition[8–11]. First, some AChRs redistribute in the plane of the membrane, moving from the extrasynaptic to the synaptic pool. At present, there is no evidence that this movement is vectorial; instead, randomly diffusing AChRs seem to become trapped when they reach an aggregate[12,13]. Second, after clustering, the metabolic stability of AChRs increases: AChRs on embryonic myotubes have a residence time in the membrane of ~1 day, whereas the residence time of adult, synaptic AChRs is ~14 days[8]. Third, a few myonuclei are always closely associated with the postsynaptic membrane, and these become transcriptionally specialized as or soon after synapses form, expressing genes that encode AChR subunits, and some other postsynaptic components, at far higher rates than non-synaptic nuclei[14,15]. This preferential transcription of AChR genes leads to an accumulation of AChR mRNA in synaptic areas, and therefore to a localized synthesis and assembly of AChRs. Fourth, after the transcriptional specialization of synaptic nuclei, AChR gene transcription is suppressed in non-synaptic nuclei, leading to an accentuation of localized synthesis. Together, these four processes explain much of the synaptic accumulation of AChRs, but further processes, such as local translational regulation, might also occur.

Neural control of postsynaptic differentiation

The precise apposition of postsynaptic specializations to the motor nerve terminal might arise in one of two ways: the motor nerve might induce AChR clusters, or clusters might form aneurally and then be recognized by the ingrowing axon. The finding that 'spontaneous' AChR clusters, often called 'hot spots', form on myotubes cultured without neurons seemed to support the latter idea. However, decisive evidence for the neural induction of AChR aggregates came from studies of nerve–muscle co-cultures carried out by Anderson and Cohen in the frog[16], and by Frank and Fischbach in the chick[17]. Both groups asked whether neurites preferentially contacted hot spots on myotubes, and both found that they did not. Instead, neurites seemed to contact myotubes at random, then new AChR clusters formed at sites of contact. Other important results of these and related studies[18–20] include the following. First, nerve-induced clusters differ in shape from spontaneous ones, in that they correspond to the geometry of the intercellular contact. Second, pre-existing clusters are stably maintained in the absence of nerves, but disperse once a genuine synapse forms. Third, many of the membrane, matrix and cytoskeletal components that are found at the postsynaptic membrane *in vivo* are co-localized with AChRs, even at spontaneous hot spots. And fourth, innervation not only alters the distribution of AChRs, but also increases their synthesis. Together, these results indicate that myotubes can generate postsynaptic components on their own, but that motor axons can localize and augment them to form a postsynaptic apparatus at the site of nerve contact.

One of the first issues to be addressed was whether the neural influence was synaptic activity. For example,

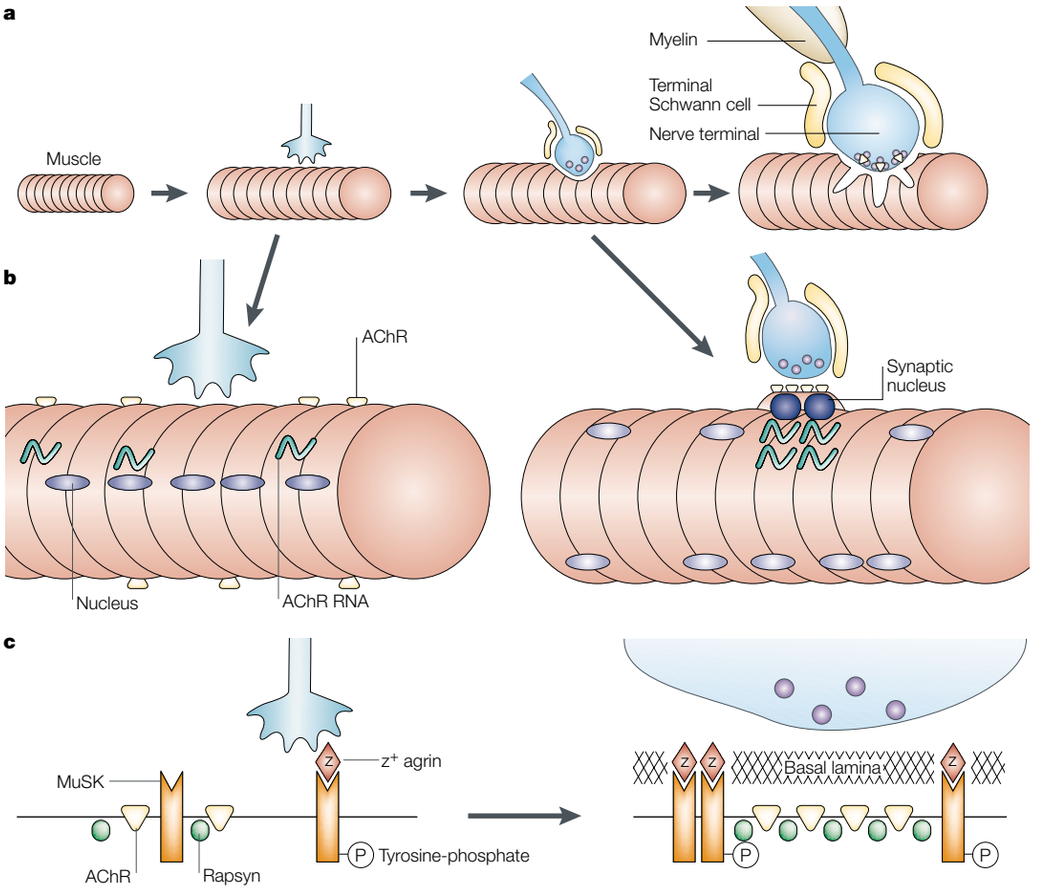

Figure 2 | **Clustering of AChRs as the neuromuscular junction forms. a** | Outline of synaptogenesis. The motor axon approaches a newly formed myotube. At the area of contact, the axon differentiates into a motor nerve terminal that is specialized for transmitter release, Schwann cell processes cap the terminal, and the muscle forms a complex postsynaptic apparatus. **b** | Acetylcholine receptors (AChRs) are initially present at a moderate level throughout the myotube surface. In adult muscle, by contrast, AChRs are highly concentrated in the postsynaptic membrane and virtually absent extrasynaptically. This clustering involves both redistribution of AChR proteins, and localized synaptic synthesis of AChRs. The local synthesis results from enhanced transcription of AChR genes by subsynaptic nuclei and by repression of extrasynaptic nuclei. **c** | The agrin–muscle-specific kinase (MuSK)–rapsyn–AChR pathway. z^+ agrin is released from the nerve terminal and becomes stabilized in the basal lamina of the synaptic cleft. Agrin activates MuSK to cluster AChRs through the cytoplasmic linker protein rapsyn.

LAMININ
Glycoprotein that is the main constituent of basement membranes. It mediates the attachment, migration and organization of cells into tissues during development.

MIDKINE
A heparin-binding growth factor of the transforming growth factor-β superfamily. Midkine was originally described as being associated with tooth morphogenesis induced by epithelial–mesenchyme interactions.

PLEIOTROPHIN
A heparin-binding mitogenic protein that induces process extension in neurons and osteoblasts.

HEPARAN SULPHATE
A glycosaminoglycan that consists of repeated units of hexuronic acid and glucosamine residues. They usually attach to proteins through a xylose residue to form proteoglycans.

acetylcholine might be the organizing agent, local depolarization might mark the site for AChR aggregation, or ion fluxes might be a precondition for other agents to act effectively. In fact, AChRs cluster at nerve–muscle contact sites in the presence of pre- or postsynaptically acting blockers of activity, showing that the process does not require electrical activity, although it might well be modulated by it[8–10]. By contrast, extrasynaptic suppression of AChR synthesis is an activity-dependent process, driven by neurally evoked muscle action potentials that trigger a signal transduction cascade involving Ca^{2+}, protein kinase C, and transcriptional regulators such as myogenin. This process, which serves as an accessible model for the widespread and important phenomenon of activity-modulated gene expression, has been studied in detail[10], but it is only tangentially related to the formation of the synaptic membrane, and we will not consider it further here.

The agrin–MuSK–rapsyn–AChR pathway

On the basis of the idea that motor neurons use chemical messengers to organize the postsynaptic membrane, many groups sought molecules that can cluster AChRs on, or increase AChR synthesis in, cultured myotubes. Several active agents were identified, including neuregulin, transferrin, collagen, LAMININ, ascorbic acid, calcitonin-gene-related peptide (CGRP), MIDKINE, fibroblast growth factor, PLEIOTROPHIN and agrin[21–28]. Many of these were reasonable candidate neural signals, given their bioactivities *in vitro* and their localizations *in vivo*. However, only for agrin is there strong evidence for a direct role *in vivo*.

Agrin, isolated by McMahan and colleagues, is a large HEPARAN SULPHATE proteoglycan that is synthesized by motor neurons, transported down motor axons, and released from nerve terminals, where it stably associates with the basal lamina of the synaptic cleft[28–30]. Agrin was identified by virtue of its ability to induce the aggregation

DOMINANT NEGATIVE
Describes a mutant molecule that is capable of forming a heteromeric complex with the normal molecule, knocking out the activity of the entire complex.

of AChRs on cultured myotubes, but was soon found also to aggregate numerous other components of the postsynaptic apparatus[31]. These results led to the 'agrin hypothesis', which states that agrin is a main nerve-derived organizer of postsynaptic differentiation at the NMJ[28] (FIG. 2c). Over the past five years, gain- and loss-of-function studies have provided strong support for this hypothesis. First, postsynaptic differentiation is profoundly impaired in agrin-deficient mutant mice[32]. Second, introduction of agrin into denervated muscles (by injection of an expression vector or the recombinant protein itself) elicits formation of a remarkably complete postsynaptic apparatus[33–35]. Third, although myotubes and Schwann cells also express agrin, alternatively spliced forms known as z^+ agrin, which are ~1000 times more potent than z^- forms *in vitro*, are expressed only by neurons[36,37]. Fourth, selective genetic disruption of the z^+ agrin form is as deleterious to NMJ formation as the null allele, but chimeric synapses made by agrin-positive neurons on agrin-deficient myotubes seem to be normal[38]. Together, these results leave us in little doubt that, at least in the context of a muscle fibre, nerve-derived z^+ agrin is crucial for postsynaptic differentiation.

Given the central role of agrin, it was important to find out how it signals, a quest that required the identification of its receptor(s). It rapidly became apparent that numerous molecules on the myotube surface were able to bind agrin and/or affect agrin signalling. These included αvβ1 and α7β1 integrins, neural cell-adhesion molecule (NCAM), laminins, pleiotrophin, *N*-acetylgalactosaminyl-terminated glycoconjugates, dystroglycan, heparan sulphate proteoglycans and MuSK[25,39–51]. As for the putative organizers themselves, their localizations and/or bioactivities made many of these molecules reasonable candidate receptors. And once more, as for agrin, loss-of-function mutations proved to be crucial in showing a central role for one of them — MuSK.

MuSK is a transmembrane receptor tyrosine kinase that was identified by virtue of its selective expression in *Torpedo* electric organ[46] and mammalian muscle[47]. Unexpectedly, muscles in MuSK-null mutant mice showed no detectable signs of postsynaptic differentiation, despite normal levels of AChR expression[52]. This observation immediately indicated that MuSK was the agrin receptor. Supporting data were soon collected, including the following: application of agrin to myotubes leads to the rapid activation of MuSK[53]; DOMINANT-NEGATIVE MuSK blocks agrin signalling in wild-type myotubes[54]; $MuSK^{-/-}$ myotubes are completely unresponsive to agrin[55]; and chemical cross-linkers covalently attach agrin to MuSK[53]. By contrast, myotubes that lack other candidate agrin receptors tested so far (laminin α2, α4, α5, β2 or γ1; integrin αv, α7 or β1; NCAM, dystroglycan and agrin itself) all bear NMJs *in vivo* and/or are agrin-responsive *in vitro* (REFS 40,56–59 and J. R. S., unpublished observations). Moreover, MuSK is selectively activated by z^+ agrin, which aggregates AChRs, whereas other binding partners discriminate little between z^- and z^+ agrin. Likewise, the ability of agrin to activate MuSK and to aggregate AChRs maps to the same carboxy-terminal 20-kDa fragment, whereas other receptors bind elsewhere[36,37,60]. Together, these results not only establish MuSK as a crucial component of the agrin signal-transducing receptor, but also indicate that other binding partners have modulatory rather than central roles. Completely satisfactory gain-of-function tests of the roles of MuSK have not been reported as yet. However, the activation of MuSK by activating antibodies *in vitro*[61,62], and the introduction of a constitutively activating mutation *in vivo*[63], induce AChR clustering in the absence of agrin, consistent with the idea that MuSK acts downstream of agrin.

A crucial effector of postsynaptic differentiation downstream of MuSK is rapsyn, a 43-kDa membrane-associated cytoplasmic protein. Rapsyn was isolated by virtue of its tight association with AChRs. It is present at NMJs as soon as AChRs cluster, co-distributes perfectly with AChRs at adult NMJs, and is present at a 1:1 stoichiometry with AChRs in the electric organ[64,65]. For rapsyn, both loss- and gain-of-function tests have given consistent results. Co-expression of AChRs and rapsyn in any of several heterologous (non-muscle) cells leads to the formation of AChR–rapsyn co-clusters, whereas AChRs are diffusely distributed when expressed on their own[66,67]. Conversely, no AChR clusters form at all on the muscles of rapsyn-null mice, or on myotubes isolated from the mutants and treated *in vitro* with any of a variety of clustering agents[68]. So, rapsyn seems to be necessary for all forms of AChR clustering.

Are AChRs only the object of clustering, or do they have a more active role in the process? In non-muscle cells, rapsyn and other synaptic components can cluster in the absence of AChRs, consistent with the idea that receptors could occupy slots in a scaffold that they do not help to shape[66,67,69,70]. However, several results indicate that the situation in muscle cells is more complex. Rapsyn fails to cluster at synaptic sites in zebrafish mutants that lack AChRs[71]. Likewise, several synaptic components that normally co-cluster with AChRs in agrin-treated myotubes fail to cluster in a variant lacking AChRs[11,72]. In mutant mice that lack an adult AChR subunit (described below), the postsynaptic membrane undergoes a profound reorganization in which levels of several membrane and cytoskeletal components decline in parallel with AChR loss, and the residual material forms small islands[73]. So, the AChR might be an active participant in the agrin–MuSK–rapsyn–AChR pathway.

Core programmes and square pegs

Over the past few years, it has become popular to view groups of regulatory molecules as 'core programmes' of development. What does this mean? In our view, to be called a core programme, the members of the group need to be epistatically related (that is, to work in a pathway), to be necessary (as shown by loss-of-function tests) and to be sufficient (as shown by gain-of-function tests). By these criteria, agrin, MuSK, rapsyn and AChRs might constitute a core programme of postsynaptic differentiation at the NMJ (FIG. 2c). At best, core programmes provide ways of evaluating data, focusing on crucial elements, and discriminating these from influences of modulators that are more peripherally

involved. At worst, core programmes can serve as a flawed rationale for forcing data into a particular framework, or for ignoring valid data that fail to fit — in other words, for pounding square pegs into round holes. In the remainder of this essay, we summarize some complex aspects of postsynaptic differentiation at the NMJ, asking whether or not they are consistent with the agrin–MuSK–rapsyn pathway.

Agrin and ARIA

The synaptic accumulation of AChRs results not only from the clustering of receptor proteins, but also from the selective transcription of AChR genes by synapse-associated myonuclei (FIG. 2b). ARIA (AChR-inducing activity), an isoform of the secreted growth factor neuregulin 1, was isolated in a search for neural factors that stimulate AChR synthesis by myotubes[21,74]. Like agrin, neuregulin is expressed by motor neurons and secreted into the synaptic cleft. And, like MuSK, neuregulin receptors are transmembrane tyrosine kinases (ErbB kinases) that are concentrated in the postsynaptic membrane at the NMJ[75–79]. These parallels led to the idea that neuregulin and agrin act as twin nerve-derived signals, with agrin organizing AChR clustering and neuregulin locally activating AChR transcription. Consistent with this idea, recombinant agrin has no effect on AChR levels in some assays, and only weak effects in others[80,81].

Unfortunately, attempts to find a direct role for neuregulin in synapse formation *in vivo* have met with little success so far. Mutant mice that lack neuregulin or ErbB2 die at early embryonic stages, before muscles form, so their synaptic phenotypes cannot be assessed. Genetic tricks (transgene expression targeted to the heart, deletion of a neuron-specific isoform, or CONDITIONAL MUTAGENESIS) can be used to circumvent embryonic lethality, and mice lacking ErbB3 or ErbB4 have been analysed as late embryos. In all of these cases, the results are mixed: there are severe neuromuscular defects, but at least some transcriptional specialization of synaptic nuclei occurs[82–85]. The interpretation of these phenotypes is complicated because neuregulin–ErbB signalling is essential for the proliferation, migration and survival of Schwann cells; these cells develop poorly or not at all in its absence[86]. Because axons receive guidance and trophic support from Schwann cells, this disruption leads to the defasciculation and eventual degeneration of motor axons. In short, there are marked neuromuscular defects in these mutants, but it is not clear whether any of these defects results from a loss of neuregulin signalling from nerve to muscle. Likewise, heterozygous neuregulin mutant adults have decreased AChR levels at their NMJs[87], but this might arise indirectly from a subtle perturbation of axon–Schwann cell interactions.

A further complication is that the localized transcription of AChRs is greatly reduced in agrin mutants[32], and is stimulated near agrin deposits that are introduced into denervated muscle[77]. Nerve-associated transcriptional specializations are also absent from MuSK$^{-/-}$ muscles[52], but present in rapsyn$^{-/-}$ muscles[68].

CONDITIONAL MUTAGENESIS
The generation of mutant animals in which the mutation can be selectively targeted to specific organs (or cell types within an organ) or induced at a specific developmental stage.

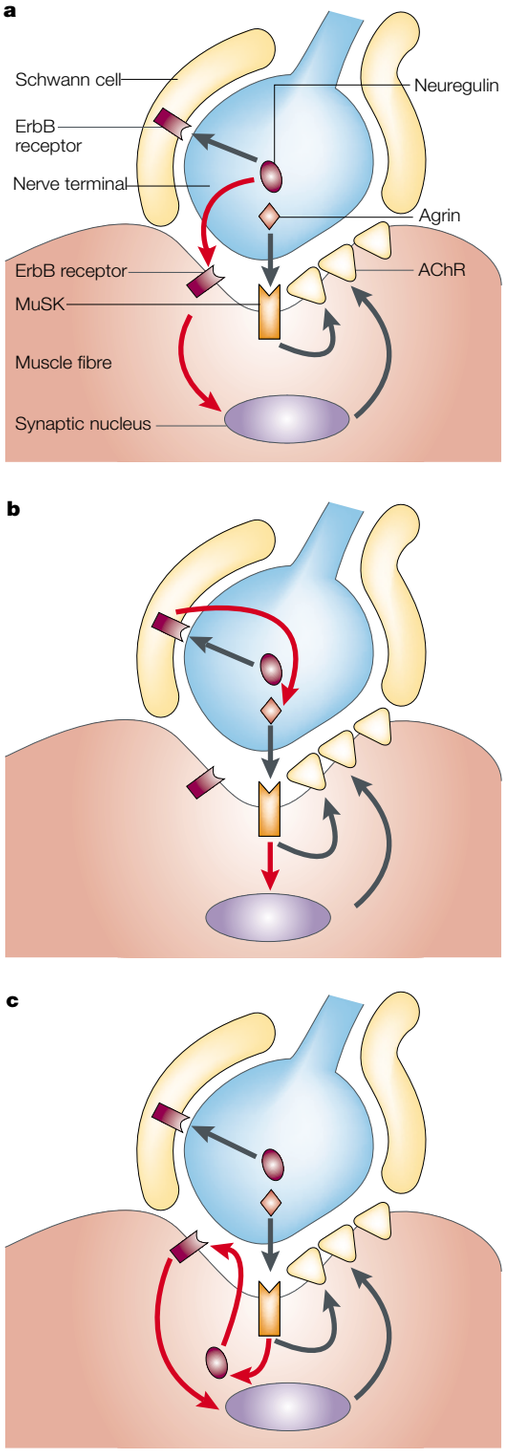

Figure 3 | **Possible roles for neuregulin in postsynaptic differentiation. a** | Neuregulin could act in parallel with agrin; neuregulin might induce acetylcholine receptor (AChR) transcription and agrin might direct AChR clustering. **b** | Neuregulin signalling from the axon is essential for Schwann cell survival, and Schwann cells are essential for axonal maintenance. In the absence of Schwann cells, axons might be unable to present agrin adequately. **c** | Agrin might direct an autocrine pathway in which muscle-derived neuregulins potentiate or mediate effects on AChR transcription.

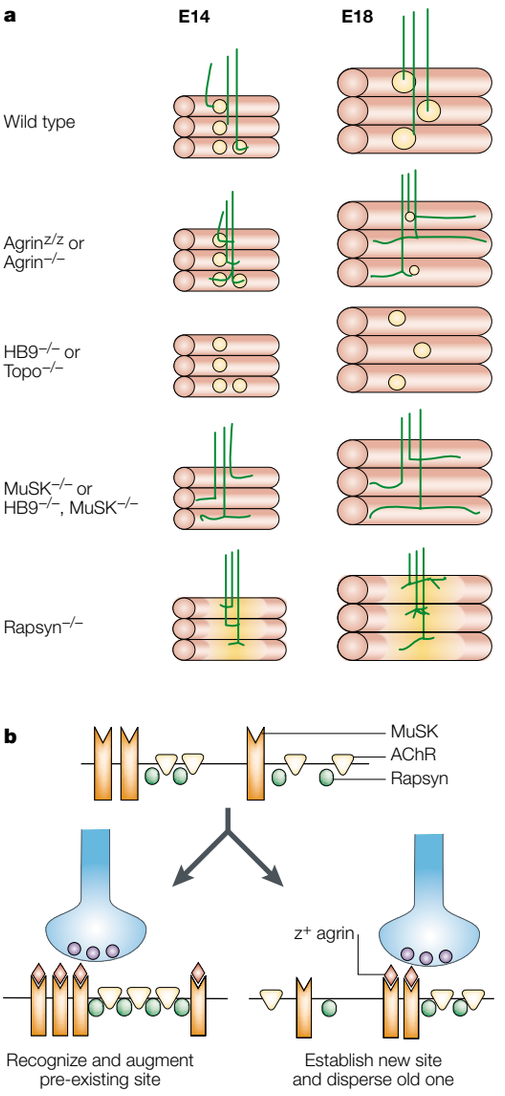

Figure 4 | **Genetic analysis of early events in AChR clustering. a** | In newly innervated wild-type muscles (embryonic day (E) 14), some axons terminate on acetylcholine receptor (AChR)-poor parts of the myotube surface and some AChR clusters are unoccupied by nerves. By E15 (E18 is shown here), the apposition of pre- and postsynaptic specializations is markedly improved. AChR clusters form normally in mutants lacking z^{+} agrin, all forms of agrin, or phrenic motor axons (these mutants lack DNA topoisomerase-IIβ (Topo) or the motor-neuron-specific transcription factor HB9, both of which are required to form the phrenic nerve through which motor axons ordinarily reach the diaphragm muscle). So, the initial steps of postsynaptic differentiation can be agrin- and even nerve-independent. Later, clusters become smaller, dimmer and fewer in agrin mutants, but persist in aneural muscle. So, axons convey an agrin-independent signal that disperses AChR clusters that have not been induced or stabilized by agrin. No AChR clusters form in mice lacking muscle-specific kinase (MuSK) or rapsyn, but localized AChR transcription persists in the absence of rapsyn. **b** | Although AChR clusters can form in the absence of agrin, it is not clear whether axons recognize these clusters and use agrin to stabilize and augment them, or whether axons use agrin to induce new clusters and a second signal to disperse the aneural ones.

It is therefore possible that agrin–MuSK signalling activates two pathways: a rapsyn-dependent pathway for clustering AChRs and a rapsyn-independent pathway for localized transcription.

How, then, might neuregulin–ErbB signals interact with agrin–MuSK signals to affect postsynaptic differentiation? Three scenarios are sketched out in FIG. 3. First, axonal neuregulin might act in parallel with agrin, with MuSK- and ErbB-initiated signals interacting intracellularly, such that both pathways must be activated *in vivo*, even though neuregulin signalling alone can activate AChR transcription in the absence of agrin or MuSK*in vitro*[88]. Second, neuregulin might act primarily as a signal from axons to Schwann cells; in its absence, Schwann cells are absent, and nerve terminals become so compromised that they cannot properly present agrin to myotubes. A third possibility is that neuregulin and ErbB kinases act in an AUTOCRINE fashion, essentially as second messengers to nerve-derived agrin. Several observations support this scheme: muscles and axons synthesize neuregulin[76]; agrin can cluster neuregulin and ErbB kinases in the absence of nerves[77]; dominant-negative forms of ErbB kinase[89] and its target transcription factor, GA-repeat-binding protein[90], can inhibit the agrin-induced local synthesis of AChRs; and synaptic nuclei become transcriptionally specialized in mice that retain muscle neuregulin but lack motor-neuronal neuregulin[85] (see REFS 9,15 for reviews of the regulation of transcription by neuregulin).

Before agrin

In its simplest form, the agrin hypothesis predicts that the secretion of agrin from the motor nerve terminal initiates postsynaptic differentiation. However, some observations made 20 years ago seemed to be inconsistent with this view: spontaneous hot spots form on cultured aneural myotubes (as mentioned above) and aneural AChR aggregates appear *in vivo*, often in an end-plate band, when motor nerves or motor neurons are ablated in rodent and duck embryos[91,92]. The former results were generally viewed as peculiarities of the culture system, and the latter ones as artefacts of incomplete denervation. Reports of near-complete or total absence of AChR aggregates in aneurally developing chick and fish muscle were consistent with the idea that results obtained *in vivo* were artefacts[93–95]. Recently, however, several groups have replicated Harris's observation[92], using genetic methods to delete motor neurons[85,96–99], and it is no longer possible to ignore it.

Particularly pertinent is the observation that, during the brief period when synaptogenesis begins in mouse muscle, some AChR clusters are not apposed by nerves and some nerve endings are not apposed to AChR clusters (FIG. 4a). One possible explanation for this observation is that secreted agrin might initially be poorly associated with the matrix, and therefore able to act at a distance[93]. Alternatively, axons might remodel rapidly at early stages, first inducing and then abandoning small AChR aggregates. However, similar 'mismatches' were also seen in mice lacking agrin (FIG. 4a). This indicated that nerves might provide a second signal that acted in

AUTOCRINE
An agent that acts on the cell that produced it.

parallel with or even before agrin. But AChR aggregates appeared even in muscles that were never innervated, owing to the genetic perturbation of their motor neurons (FIG. 4a). So, myotubes can form AChR aggregates on their own *in vivo* and *in vitro*. Moreover, these aggregates are nearly all in a central end-plate band, throwing into doubt the idea[3] that the location of synapses is entirely determined by the site at which the motor nerve enters the muscle.

Another surprise came from the comparison of agrin-free and aneural muscles later in embryogenesis. AChR aggregates were few in number and small in size in the absence of agrin, but large and numerous in the absence of motor nerves (FIG. 4a). It therefore seems that axons do provide a signal other than agrin, but that this signal is inhibitory rather than inductive, leading to dispersal of those AChR aggregates that have not been formed or stabilized by agrin. This second signal might be synaptically evoked electrical activity, which is known to downregulate AChR synthesis (see above), but several observations indicate that an activity-dependent dispersal signal also exists[100,101].

These unexpected observations raise two new questions. First, why do aneural AChR aggregates, the apparent *in vivo* correlates of spontaneous hot spots, form (in many muscles at least) preferentially in an 'end-plate' band? Four further observations are pertinent: no aggregates at all form in rapsyn$^{-/-}$ or MuSK$^{-/-}$ muscles, whether or not they are innervated; the centre of each myotube is older than its ends, because myotube formation begins at the centre and proceeds by successive waves of myoblast fusion at the ends; levels of AChR mRNA are higher in the central end-plate band of aneural muscle than at the periphery; and although MuSK activity is stimulated by agrin, MuSK is active at a low level in the absence of agrin[55]. Together, these results prompt the hypothesis that the central regions of myotubes, being most mature, bear the highest levels of both MuSK and AChRs, and are therefore most likely to form AChR clusters.

Second, what is the role of aneural clusters in synapse formation? One possibility is that motor axons ignore these clusters *in vivo*, just as they seem to ignore hot spots *in vitro*[16,17]. Instead, they use agrin to organize new clusters, then use the 'second signal' to disperse the non-synaptic aggregates. Alternatively, axons might recognize these clusters in some cases, or encounter them by chance, and then use agrin to enlarge and/or stabilize them (FIG. 4b). An intriguing idea is that both sequences occur, with the aneural clusters serving a 'back-up' function to ensure that all myotubes eventually receive synapses. The most direct approach to distinguish between these possibilities will be to observe neurites and AChRs simultaneously *in vivo* or in explants during the initial period of synaptogenesis.

From agrin to MuSK

In some ways, agrin and MuSK are a simple growth factor–receptor pair, akin to neurotrophin–Trk kinase or insulin–insulin receptor pairs. In each of these cases, binding of the factor to the ectodomain of the receptor leads to receptor dimerization, kinase activation, recruitment of signal-transduction intermediates and generation of signals that lead to the biological response: glycolysis for insulin, suppression of apoptosis for nerve growth factor, and so on. However, agrin–MuSK signalling is unique in two ways that complicate this simple model.

First, whereas most growth factors activate their receptors by binding to them directly, it has been difficult to show direct binding of agrin to MuSK. Moreover, although agrin can activate MuSK in myotubes, it does not activate MuSK that is expressed in heterologous cells. In addition, chemical cross-linking of agrin to MuSK occurs in muscle cells or in the presence of muscle extracts, but not when agrin is applied to MuSK in non-muscle cells. Together, these results led to the idea that MuSK is complexed with another molecule that is expressed selectively in muscle and is essential for agrin binding[53]. This hypothetical entity, dubbed muscle accessory specificity component (MASC), might be a second subunit of a heteromeric complex, a binding subunit, a relatively nonspecific affinity-enhancing cofactor, or a post-translational modification. Yet another possibility, raised by recent studies of cultured myotubes, is that MASC is a particular carbohydrate present on several molecules, including agrin, where it is linked to an amino-terminal site[102]. When long fragments of agrin that contain this sugar are used to activate MuSK, no accessory cofactors are needed. In most studies, however, shorter fragments have been used; in these cases, related glycoconjugates on the myotube surface function as MASC. At present, this intriguing idea awaits critical testing.

Second, whereas most growth factors use their receptors only to elicit a biological response, agrin also needs to localize its response precisely beneath the nerve terminal. A mismatch of even a few micrometres would be detrimental to fast synaptic transmission, yet AChR clusters induced by diffusible intracellular messengers might occur well beyond the sites at which agrin acted. A possible solution was indicated by two seemingly contradictory observations. On the one hand, rapsyn and MuSK co-cluster when co-expressed in heterologous cells, just as rapsyn and AChRs do[70,71]. On the other hand, MuSK itself clusters beneath nerve terminals in rapsyn-deficient mice, whereas AChRs and other components of the postsynaptic membrane do not[67,70,76]. These results indicate that MuSK might use rapsyn in two different ways: by promoting the AChR–rapsyn association to cluster AChRs, and by interacting with rapsyn to recruit AChRs to a MuSK-containing scaffold. Consistent with the idea that these functions are distinct, mutant MuSK proteins that are not able to interact with rapsyn in heterologous cells can induce the formation of AChR–rapsyn aggregates in myotubes[55]. Moreover, beads coated with recombinant z^+ agrin fragments (but not soluble agrin) can induce the clustering of kinase-deficient MuSK mutants that are unable to aggregate AChRs (H. Zhou and J. R. S., unpublished observations). So, the nerve might use MuSK in separate ways to induce and localize the AChR aggregate. One possibility is that only cell- or matrix-associated agrin can cluster MuSK by a

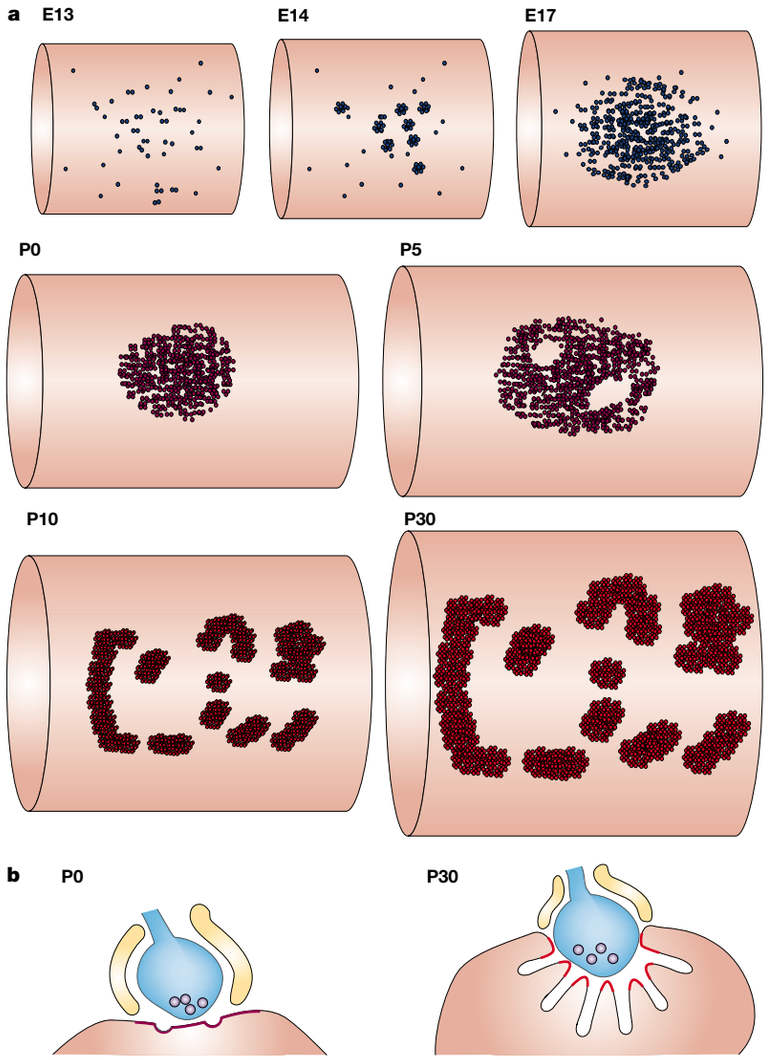

Figure 5 | **Maturation of the postsynaptic apparatus. a** | Acetylcholine receptor (AChR) microclusters coalesce to form a loose aggregate. Late in embryogenesis, the aggregate consolidates to form a plaque: its borders sharpen, its length decreases, and AChR density increases. Postnatally, the plaque becomes perforated to eventually form a pretzel-like array of branches. The branches then expand in an intercalary fashion as the muscle grows. Change in AChR colour denotes the switch from γ- to ε-containing AChRs. **b** | As these changes occur, the plaque is indented to form a gutter, then invaginated to form folds. AChRs are concentrated at the crests of the folds. E, embryonic day; P, postnatal day.

kinase-independent mechanism, whereas both bound and soluble agrin can activate MuSK kinase activity to produce the AChR cluster. It is also possible that other ligands can cluster or activate MuSK.

From MuSK to rapsyn

One might imagine that signalling from MuSK to rapsyn, which presumably shares features with other tyrosine-kinase-initiated signal-transduction pathways, would be well understood. Nothing could be further from the truth. A few groups have begun to identify active sites on the cytoplasmic domain of MuSK and proteins that interact with these sites[55,103–105]. Other groups have sought MuSK substrates and potential downstream kinases (see below). However, overall, this work is still in its infancy, and the signalling pathways have not yet reached rapsyn. Here we mention two aspects of rapsyn function that might be important as this work proceeds.

First, rapsyn might act in part by linking AChRs to the cytoskeleton. Early studies showed that cytoskeletal proteins, such as actin and spectrin, are closely associated with AChR clusters, and that drugs that disrupt the cytoskeleton, such as cytochalasin, prevent cluster formation and/or disperse pre-existing clusters[106]. Agrin could then promote or strengthen this linkage through MuSK. Several lines of evidence support this view. In the presence of agrin, AChRs are less prone to extraction from the membrane by detergents, indicating that agrin enhances their association with the cytoskeleton[107]. Agrin induces the polymerization of actin at sites of AChR clustering, and clustering does not occur when this polymerization is blocked[108]. Moreover, RHO-GTPASES (Rac and Cdc42), which are known to mediate reorganization of the actin cytoskeleton in many systems, are activated by agrin and are crucial for agrin-induced AChR clustering[109]. Further support comes from an observation of developing NMJs in culture: motor axons induced the formation of microvilli, FILOPODIA and ruffles at sites of contact with myotubes. Soluble z^+ agrin had a similar, although less-pronounced effect, and similar processes were observed at nascent synapses *in vivo*[110]. Such microprocesses are generally signs of Rho-GTPase-mediated, actin-dependent motile activity. This motile response provides an independent line of indirect evidence that agrin can locally modulate the actin cytoskeleton, and also raises the possibility that this modulation could be involved in some of the late, topographic changes in the postsynaptic apparatus, which are discussed below.

Second, rapsyn can cluster AChRs when both are expressed in any of several non-muscle cells that lack agrin and MuSK[66,67,69,70]. By contrast, not even spontaneous (agrin-independent) AChR clusters form in MuSK-deficient myotubes[55], and overexpression of rapsyn in wild-type myotubes induces little, if any, additional cluster formation[111,112]. These observations raise the possibility that muscles contain an endogenous activity that prevents rapsyn clustering, and that MuSK-initiated signalling relieves this inhibition. Agents that can induce AChR clustering even in the absence of MuSK, such as laminin 1 and some plant LECTINS, might block this inhibitory influence, acting either downstream of MuSK or in a parallel pathway[88,113–115]. To our knowledge, experiments have not yet been designed to seek a muscle-specific factor that prevents AChR clustering when MuSK is absent or inactive.

Synaptic maturation: beyond the core

NMJs are functional even before birth, and neonates are completely dependent on neuromuscular transmission for survival, yet the postsynaptic membrane of the neonate is very different from that of the adult. Transformation of the nascent receptor cluster into the adult postsynaptic apparatus involves a complex set of processes that occur over a protracted period. There are at least five important sets of alterations[3]. First, the shape:

RHO-GTPASE
A Ras-related GTPase that is involved in controlling the polymerization of actin.

FILOPODIA
Long, thin protrusions at the periphery of migrating cells and growth cones. They are rich in bundles of F-actin.

LECTINS
Sugar-binding proteins that tend to agglutinate cells. Concanavalin A is a widely used example.

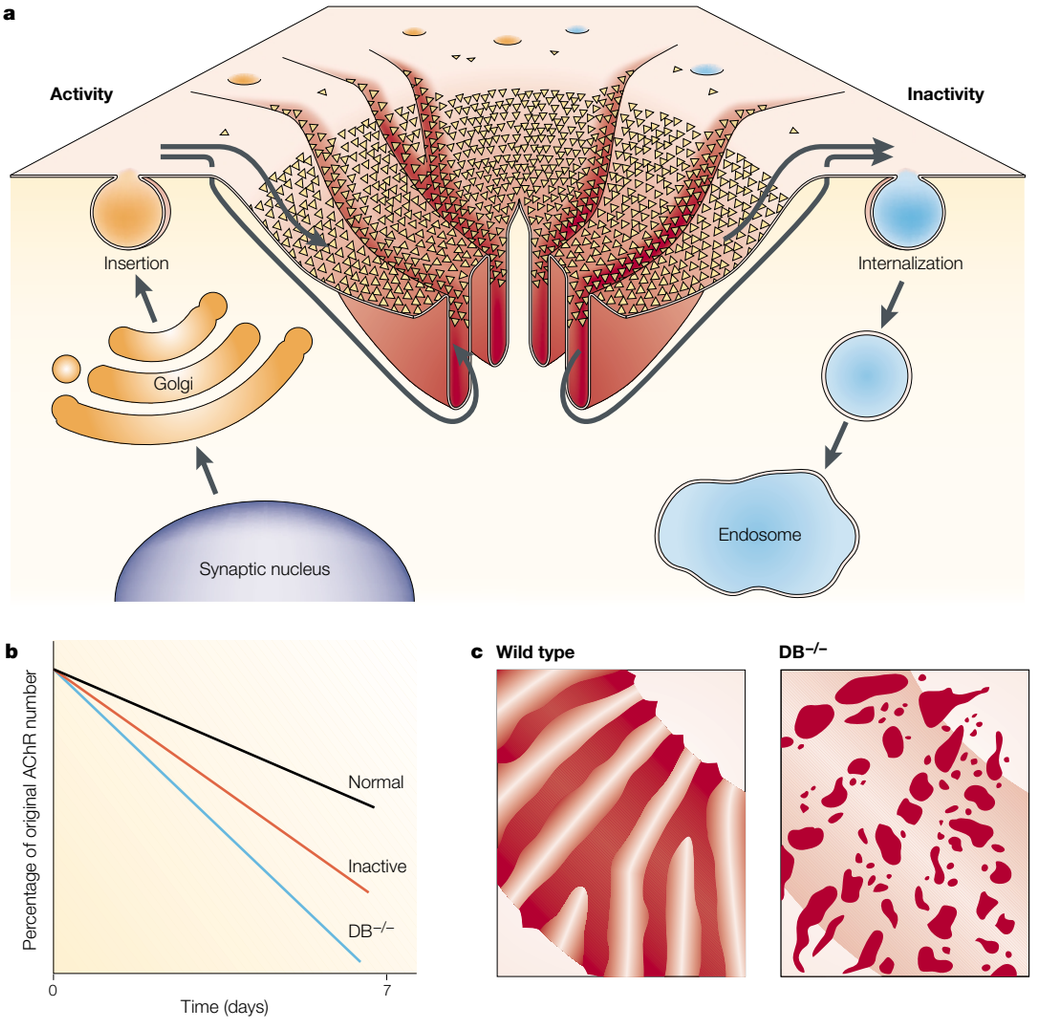

Figure 6 | **Turnover of AChRs in active, inactive and mutant muscles. a** | Three-dimensional view of a bouton, showing acetylcholine receptors (AChRs) at the crests of folds. In one model, new AChRs are added by exocytosis and old AChRs are removed by endocytosis, both in perisynaptic areas. The folds provide a route by which AChRs could enter or leave the dense patch. Arrows show processes that are believed to be affected by activity in adult muscle. **b** | AChR turnover (loss) is slow from normal, active NMJs, but accelerated by paralysis and by knocking out dystrobrevin (DB) (M. Akaaboune, R. M. Grady, J. R. S. and J. W. L., unpublished observations). **c** | *En face* views of a bouton in control and α-dystrobrevin mutant mice. Striations in the control are folds. In the mutant, AChRs break up into granular microclusters.

the junction changes from a simple oval plaque to a pretzel-like set of branches (FIG. 5a). Second, the topography: the junctional membrane changes from a flat sheet to an invaginated surface with gutters and folds (FIG. 6a). Third, the extracellular matrix and cytoskeletal constituents: the composition of the basal lamina that overlies the AChR-rich membrane, and the cytoskeletal apparatus that underlies it, change quantitatively and qualitatively. Fourth, channel function: a shift in AChR subunit composition leads to a change in its Ca^{2+} permeability. Fifth, molecular partitioning: ion- and ligand-gated channels, along with their associated cytoskeletal elements, segregate into discrete alternating domains (FIG. 5b). Equally significant changes take place on the presynaptic side of the maturing NMJ: terminals become more efficacious, motor axons myelinate, and at least half of the axonal branches are trimmed as synapse elimination removes all but one axon at each NMJ. Most studies of these late events have focused on mouse and rat, but similar changes have been described in chicks, arguing for some generality in the maturation at vertebrate NMJs[116].

Knowing why something occurs is a big help in understanding how it occurs[117]. Maturation of the synapse is presumably required for the increasing functional demands on this synapse as muscle fibres grow, and animals require rapid responses from their motor systems without sacrificing fine-tuning. The fact that neurotransmitter release becomes more reliable, and both receptor density and total receptor number increase[3], support this view. The continued postnatal increase in the 'safety factor' (the surplus of AChRs beyond what is needed to achieve muscle excitation after nerve stimulation[118]) might, among other things, make maturing mammals less susceptible to

paralysis from snake toxins such as BTX. In this view, the maturational changes are a means of providing life-long potentiation of this synapse. On the other hand, it is not obvious why these late steps require weeks to complete when a functional synapse can be constructed so quickly. Perhaps the protracted postnatal maturation reflects the complex feedback required to fine-tune the system as the animal first begins to work against gravity, and then gradually changes in mass and shape. If so, many of these later steps might be pertinent to central synapses, the efficacy of which is famously experience dependent.

One important question concerning neuromuscular maturation is whether these later steps are downstream of the molecular cascade initiated by agrin, or controlled by completely separate pathways. Ectopically expressed agrin can induce postsynaptic structures with a surprisingly mature appearance[33,34,77,119], arguing that one nerve-derived signal can trigger a multistep postsynaptic programme. However, synaptically evoked activity seems to affect late steps in profound ways.

From plaque to pretzel

The overall shape, the three-dimensional topography and the molecular domains of the postsynaptic membrane undergo marked changes, from the time at which nerve-associated AChR clusters are first recognizable to the time at which the NMJ is mature (FIG. 5a). The alterations in shape occur in several phases, beginning with a phase in which the number of AChRs beneath the nerve terminal increases. Then, during a period of embryonic consolidation, the length of the AChR cluster decreases in the days before birth[120]. This decrease corresponds to a change in the border of junctions and an increase in AChR density. In embryonic life, the borders of the AChR cluster are initially shaggy. As the edges of the synapse become better defined and AChR density increases, the cluster becomes more compact, indicating that this phase comprises a physical consolidation. By birth, the consolidation is complete and the junctional AChRs form well-delineated, oval-shaped plaques. Then, in early postnatal life, the plaque perforates as AChRs are lost from regions within it. As this process continues, the plaque transforms into a series of branches, giving the junction a pretzel-like appearance[120–123]. Finally, as the animal grows and muscle fibres enlarge, the AChR pattern grows in an intercalary way, without any change in the number or arrangement of the branches[124,125]. This expansion in the size of the postsynaptic region is probably related to an increase in the muscle fibre surface area, and might reflect new membrane insertion throughout the growing muscle. Consistent with this mechanism, the number of AChRs at the synapse increases, but AChR density remains constant, during the intercalary enlargement.

The spatial alignment between nerve terminals and AChR clusters is also modified during the maturation process. At birth, the nerve and the cluster are imperfectly aligned[126]. At many neonatal NMJs, nerve sprouts and Schwann cell processes extend beyond the perimeter of the plaque to overlie AChR-poor membrane. At the same time, some regions of each AChR plaque are not occupied by nerve terminals; occupied and unoccupied regions are indistinguishable in AChR density. This imprecise alignment indicates that the stereotyped oval shape of the AChR cluster is specified by programmes in the muscle rather than by local nerve signalling. This idea is supported by the plaque-like shape of clusters that form in aneural muscles, as described above[97,98].

The loss of AChR sites that transforms the plaque into a pretzel is more peculiar. This loss occurs in parallel with a regressive presynaptic phenomenon called synapse elimination, in which all but one axonal input to each junction is removed[3]. This temporal correlation leads naturally to the idea that pre- and postsynaptic remodelling are causally related. In fact, time-lapse imaging shows that some lost postsynaptic sites are originally occupied by nerve terminals that are themselves eliminated[126]. Because the plaque is never completely overlaid by nerve terminals, other sites might be lost because they are unoccupied. Moreover, nerve terminals are highly dynamic in neonatal junctions (M. Walsh and J. W. L., unpublished observations), whereas, later in life, they are immobile, at least in some muscles[124,125]. These results indicate that AChRs might be selectively retained at sites of stable nerve–muscle contact. Studies of adult NMJs indicate a mechanism for this loss — AChR sites that are not activated by acetylcholine are preferentially removed from junctions at which other sites are active[127]. The overall effect of this sculpting of the AChR cluster is that nerve and AChR cluster become perfectly aligned.

The third dimension

Concurrent with these changes in shape, the junction remodels in the third dimension, as the relatively flat membrane of the embryonic synapse becomes convoluted in three ways (FIG. 5b). First, the nerve essentially sinks into hemicylindrical gutters in the muscle fibre surface[123,128]. In parallel, the membrane of each gutter acquires invaginations called junctional folds, which are ~0.1-μm wide, ~1-μm deep and spaced at 1–3-μm intervals. These changes increase the contact area between nerve and muscle (the gutters), and increase the AChR density associated with release sites (the folds). Last, AChRs come to be preferentially located at the crests of junctional folds, whereas voltage-gated Na^+ channels are found preferentially at the troughs[8,119,129]. All of these changes enhance synaptic transmission from nerve to muscle.

How do gutters and folds form? One fanciful proposal is that axons adhere to sites on the basal lamina that are themselves attached to the postsynaptic membrane[123]. As muscle fibres grow, they presumably add membrane between these attachment sites. If the nerve terminal is relatively resistant to stretching, the consequence of the spread in attachment sites is to cause the muscle fibre to crawl up the sides of the axon. Likewise, localized membrane insertion, perhaps stimulated by agrin[110], could cause folding of the postsynaptic surface between nerve–muscle attachment sites.

What is the purpose of folds? One clue comes from the subdivision of the pre- and postsynaptic membranes into alternating domains with distinct compositions. In the nerve terminal, active zones (transmitter release sites) alternate with sites of preferential endocytosis, and the active zones are perfectly aligned with the mouths of junctional folds. Postsynaptically, the AChRs become partitioned to the tops of folds and partway down their sides, along with associated cytoskeletal elements such as rapsyn and utrophin. By contrast, the troughs of the folds are poor in AChRs but rich in NCAM, Na^+ channels and the cytoskeletal element ankyrin[119,129–131]. Concentrations of Na^+ channels, NCAM and associated cytoskeletal elements occur later than AChR clustering[119,122,130,131]. This arrangement ensures that AChRs are in close proximity to a membrane 'sink', where AChR mobility might be unrestricted (see below). Na^+ channels can take advantage of the synaptic depolarization induced by AChR activation, without having their current shunted by that of the AChR channels.

The mechanisms that divide folds into domains are still mysterious. Although fold-like structures form at AChR clusters in cultured myotubes[132], the invariant association of active zones in nerve terminals with fold mouths indicates a role for intercellular interactions in their formation or placement. In a simple model, the nerve–muscle attachment sites mentioned above might be most numerous or strongest at active zones. Attractive candidates for linker proteins are laminin α4 in the synaptic basal lamina, and Ca^{2+} channels in the nerve terminal. Laminin α4 is arranged with a periodicity that corresponds to folds; Ca^{2+} channels are lined up at the edges of active zones. Laminin α4 and Ca^{2+} can be isolated as components of a multimolecular complex from *Torpedo* synaptic membranes, and the apposition of folds to active zones is lost in mutant mice that lack either laminin α4 or the α_{1A}-subunit of Ca^{2+} channels (REFS 57,133 and J. R. S., unpublished observations). So, a link passing through the basal lamina from nerve to muscle could either mediate the formation of folds opposite to active zones, or bring the pre- and postsynaptic specializations into apposition once they have formed. Such a link would be useful in maintaining the alignment of these specializations during the intercalary postnatal growth mentioned above. Finally, once folds begin to form, cytoskeletal rearrangements are likely to be responsible for dividing them into domains. The presence of distinct cytoskeletal elements at crests and troughs (for example, utrophin and ankyrin), along with the finding that mouse mutants that lack components of the dystrophin–glycoprotein complex (discussed below) have reduced numbers of folds, support this view[13,58,130,134,135]. Moreover, in mice that lack just one component of this complex — α-dystrobrevin — the molecular distinctions between crests and troughs are blurred[58].

AChRs grow up

The adult postsynaptic apparatus differs from its neonatal counterpart molecularly as well as topologically. The most-studied molecular change is that of the subunit composition of AChRs. The embryonic form of AChRs, which contain γ-subunits (subunit composition $\alpha_2\beta\delta\gamma$), is replaced during the first postnatal week by AChRs that contain homologous ε-subunits ($\alpha_2\beta\delta\varepsilon$)[136–138]. This subunit switch has two opposing effects on AChR conductance: the duration of channel opening becomes shorter, but the channel conductance and its Ca^{2+} permeability increase[139]. These changes have opposite effects on current flow, so their physiological purpose is unclear. The replacement of one subunit by another might also provide a way to recruit novel cytoskeletal or signalling components as the synapse matures, but analysis of mutant mice that lack the ε-subunit has so far failed to support this possibility[73,140]. NMJs develop normally in such mutants during the early postnatal period, but the γ-subunit switches off on schedule; AChR levels decline and animals die from progressive neuromuscular weakness. So, the lethality probably says more about the inability of γ-subunit-containing AChRs to compensate numerically for the loss of ε-containing AChRs than about the functional significance of the switch.

Despite some doubt about its purpose, the mechanism of the subunit switch has attracted attention, in part because similar developmental switches have been described for both excitatory (glutamate) and inhibitory (glycine) receptors in the brain (discussed in REF. 73). It is clear that the γ-to-ε switch occurs at the transcriptional level, and regulatory elements from both genes have been characterized in transgenic mice[138,141]. However, the signals that trigger the switch are unknown. Because agrin is present well before switching occurs, this protein cannot be the sole trigger. On the other hand, application of agrin to denervated adult muscle does induce the formation of ε-subunit-containing AChRs[77]. One possibility is that the switch is part of the muscle's endogenous programme of maturation, akin to its ability to replace embryonic with adult-type myosins. Consistent with this idea, the ε-subunit appears nearly on schedule in muscles that are denervated at birth[142]. Alternatively, neuregulin–ErbB kinase signalling might be involved; indeed, neuregulins preferentially activate transcription of the ε-subunit gene *in vitro*[143].

AChRs settle down

As AChRs mature in arrangement and molecular composition, they also become more stable and less susceptible to disassembly. The lifetime of AChRs in the membrane, as assessed by the retention of radioactive BTX, is an order of magnitude longer at adult NMJs than in embryonic clusters (>1 week versus <1 day), with the change occurring during postnatal life[8]. During this period, AChR clusters become less susceptible to disassembly. For example, plaques disassemble quickly after denervation during the first postnatal week[144,145]. In adults, by contrast, synaptic AChRs remain clustered for many weeks after denervation, even after muscle fibres have become highly atrophic[146]. A similar change in susceptibility to dispersal is also evident when comparing the effects of Ca^{2+} chelators or collagenase on NMJ muscles of different ages[147].

What causes these changes? Increased metabolic stability has been suggested to be associated with the switch from γ-subunit to ε-subunit[148], but the resistance of plaques to disassembly seems to be controlled in a different way, because NMJs in adult denervated muscle contain a preponderance of embryonic AChRs, yet they are stably maintained[146,148,149]. On the other hand, both forms of stabilization — metabolic and macroscopic — seem to be related to postnatal maturation of the cytoskeleton and basal lamina. In particular, components of the dystrophin–glycoprotein complex, which links the basal lamina to the cytoskeleton, have recently been shown to be crucial.

The importance of this complex for the general maintenance of muscle integrity is shown by the fact that mutation of several of its components (including laminin in the basal lamina, sarcoglycans in the membrane, and dystrophin in the cytoskeleton) leads to MUSCULAR DYSTROPHY in humans and experimental animals. The complex is also present at the NMJ, but in a specialized form[122,131]; indeed, the finding that the transmembrane component — dystroglycan — binds agrin, led for a time to the idea that dystroglycan might be the principal agrin receptor[43–46]. We now know that this is not the case, because myotubes can cluster AChRs and are agrin-responsive in the complete absence of dystroglycan[58,59]. However, this complex is involved in the segregation of AChRs into discrete domains, as described above, indicating that agrin might act through distinct (but interacting[70]) complexes that contain MuSK and dystroglycan, respectively, to regulate formation and maturation of the synapse. In addition, the complex is involved in the maintenance or stabilization of clusters, as shown most clearly in mutant mice that lack the dystrophin-binding proteins α-dystrobrevin or α1-syntrophin. Although these mice show no (syntrophin) or mild (dystrobrevin) muscular dystrophy, their postsynaptic apparatus is not adequately maintained: AChR-rich branches fragment and, in dystrobrevin mutants, AChRs have a half-life 2–3-fold shorter than that of controls (FIG. 6b,c)[58,150].

Once formed, the postsynaptic apparatus can be maintained for the lifetime of the animal, but, importantly, it must not be immutable. To maintain its precise apposition to the nerve, it must be able to remodel over a prolonged period as the animal grows, expand or shrink with atrophy and hypertrophy, and add or retract branches as axons sprout[3,124,125]. How are growth and remodelling controlled? Tyrosine phosphorylation is a key regulatory mechanism throughout the nervous system, and there are indications that several tyrosine kinases modulate or act in parallel with MuSK to shape the AChR cluster. The AChR β-subunit is rapidly tyrosine phosphorylated after the addition of agrin. The phosphorylation requires MuSK, but is not directly carried out by MuSK, and might require kinases of the SRC family[151]. AChRs cluster even when this phosphorylation is blocked by site-directed mutagenesis, but the association of the clusters with the cytoskeleton is decreased[152–155]. Likewise, synapses seem normal in mutant mice lacking Src kinases, but AChR clusters are destabilized on myotubes cultured from these mice[156]. Furthermore, neurotrophins, acting though Trk tyrosine kinases, seem to modulate the size and shape of the postsynaptic membrane at the NMJ, a role possibly related to one they have at central synapses[157–159]. Interestingly, there are several isoforms of α-dystrobrevin in muscle, and the one most highly concentrated at the NMJ is tyrosine phosphorylated[160]. This form is more effective in rescuing the defects in the dystrobrevin mutant than the non-phosphorylatable form (R. M. Grady and J. R. S., unpublished observations), pointing to a further target of regulation.

AChRs on the move

Early studies, using the technique of FLUORESCENCE RECOVERY AFTER PHOTOBLEACHING (FRAP), showed that diffuse AChRs in cultured myotubes can move without apparent restriction in the plane of the membrane, whereas clustered AChRs are relatively immobile. Recently, it has become possible to monitor the mobility of AChRs at normal adult NMJs[161,162]. Surprisingly, junctional AChRs were found to have greater mobility than was previously suspected, and to exchange continuously with perisynaptic AChRs (FIG. 6a). Individual AChRs remain at one site within an NMJ for as little as 8 hours.

This result has two interesting implications. First, it provides a mechanism for maintaining the complex architecture of the postsynaptic structure in the face of ongoing turnover of individual AChRs. For example, junctional AChRs might leave crests by moving down the sides and along the troughs of the folds until they reach the perijunctional space to be internalized. Lacking this 'back door', AChRs might be unable to escape at plaque borders in a timely manner. Conversely, newly inserted AChRs might travel the same pathway in reverse, before they become trapped, albeit temporarily, at the tops of folds (FIG. 6a).

Second, dynamic equilibria can be regulated to provide a mechanism for altering AChR density within a macroscopically stable structure. For the NMJ, like other synapses, a crucial regulatory factor is electrical activity. Indeed, experiments carried out 20 years ago showed that AChR turnover increases markedly after denervation, is restored to normal after re-innervation, and is regulated by activity[149,163,164]. Quantitative assays of the intensity of fluorescent BTX-labelled NMJs have now allowed a re-examination of this phenomenon at higher resolution, revealing that effects of activity on AChR dynamics are rapid and highly localized[161]. It seems that AChRs trapped at the crests of folds in active synapses seldom escape to the perisynaptic region, despite significant mobility within this domain. However, within an hour after blockade of neurotransmission, AChRs migrate out of the folds into perisynaptic regions, where they are internalized, and the AChR density at the crests of folds begins to drop. For the duration of activity blockade, AChRs are lost at a rate of ~4% per hour, with no detectable addition of new AChRs. By comparison, ~9% of AChRs are lost per day in normally active muscle (~0.4% per hour), and there is no net loss because these AChRs are replaced.

MUSCULAR DYSTROPHY
A group of genetic diseases characterized by progressive weakness and degeneration of the skeletal muscles, which control movement. The main forms of muscular dystrophy include myotonic, Duchenne and Becker.

SRC
A cytoplasmic tyrosine kinase that was first identified as a transforming oncogene in an avian retrovirus. This kinase is the prototypical kinase from which Src-homology regions were first described.

FLUORESCENCE RECOVERY AFTER PHOTOBLEACHING
A method used to measure the lateral diffusion of membrane elements. It requires tagging of the molecule of interest with a fluorescent marker, photobleaching of the label with a pulse of laser light, and a subsequent measure of the rate of fluorescence recovery into the bleached area as other labelled molecules move into it.

A hypothetical scheme to explain these results is as follows: even at adult NMJs, AChRs can migrate both into and out of the synapse, but the avidity of their interaction with cytoskeletal moorings tends to retain them at the crests of folds. This avidity is tightly regulated by some consequence of postsynaptic activity, such as Ca^{2+} flux, and AChRs can quickly become untethered from their scaffold when activity is shut off. Once AChRs leave the crests of folds, they fall prey to the endocytotic machinery that surrounds this domain; they are internalized, and either recycled or degraded. It is worth noting recent interest in the idea that some forms of synaptic plasticity at central synapses might be mediated by activity-dependent alterations in the rates of neurotransmitter exocytosis and endocytosis[165]. If these phenomena in the central nervous system prove to be similar to those at the NMJ, the latter might be particularly well suited to elucidating the underlying mechanisms.

Conclusions

How far have we come and how far do we have to go? Hundreds of proteins might be involved in building a synapse, but elucidation of the agrin–MuSK–rapsyn–AChR pathway provides hope that some developmental steps might be explained by a small number of important molecules, with others playing modulatory roles. If this is so, the prospects for molecular explanation are good. It is sometimes thought that the situation at central synapses will be so complicated that the NMJ is unlikely to serve as a good model, but this is not necessarily the case. Loss of a single receptor-linking protein, gephyrin, has as devastating an effect on postsynaptic differentiation at central inhibitory synapses as does the loss of rapsyn at the NMJ[166].

It is equally clear that agrin, MuSK and rapsyn do not begin to explain the full range of events that occur as NMJs are induced, assembled, matured, modulated and maintained. But these steps can now be described, owing to the availability and combination of powerful new microscopic and genetic techniques that can, so far, be applied most readily to the NMJ (see, for example, REFS 71,161,162,167–169). These new forays are in their infancy, but there is a strong motivation to pursue them, because issues of maturation, modulation and maintenance will probably be key to understanding plasticity in the brain. There is no reason to discount the possibility that similar mechanisms will underlie these phenomena at neuromuscular and central synapses.

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