

Laminins promote postsynaptic maturation by an autocrine mechanism at the neuromuscular junction

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A prominent feature of synaptic maturation at the neuromuscular junction (NMJ) is the topological transformation of the acetylcholine receptor (AChR)-rich postsynaptic membrane from an ovoid plaque into a complex array of branches. We show here that laminins play an autocrine role in promoting this transformation. Laminins containing the $\alpha 4$, $\alpha 5$, and $\beta 2$ subunits are synthesized by muscle fibers and concentrated in the small portion of the basal lamina that passes through the synaptic cleft at the NMJ. Topological maturation of AChR clusters was delayed in targeted mutant mice lacking laminin $\alpha 5$ and arrested in mutants lacking both $\alpha 4$ and $\alpha 5$.

Analysis of chimeric laminins *in vivo* and of mutant myotubes cultured *aneurally* demonstrated that the laminins act directly on muscle cells to promote postsynaptic maturation. Immunohistochemical studies *in vivo* and *in vitro* along with analysis of targeted mutants provide evidence that laminin-dependent aggregation of dystroglycan in the postsynaptic membrane is a key step in synaptic maturation. Another synaptically concentrated laminin receptor, Bcam, is dispensable. Together with previous studies implicating laminins as organizers of presynaptic differentiation, these results show that laminins coordinate post- with presynaptic maturation.

Introduction

A hallmark of the chemical synapse is the precise apposition of its pre- and postsynaptic specializations: neurotransmitter release sites in nerve terminals lie directly opposite concentrations of neurotransmitter receptors in the postsynaptic membrane. One mechanism for ensuring this coordination is the presentation by synaptic partners of factors that locally organize each other's differentiation. At the skeletal neuromuscular junction (NMJ), for example, agrin and acetylcholine secreted from axons pattern the postsynaptic membrane, and laminins and fibroblast growth factors secreted from the muscle pattern the nerve terminal (Noakes et al., 1995; Gautam et al., 1996; Burgess et al., 1999; Patton et al., 2001; Nishimune et al., 2004; Lin et al., 2005; Misgeld et al., 2005). However, recent studies have emphasized the ability of postsynaptic components, such as acetylcholine receptors (AChRs), to aggregate in the absence of innervation (Lin et al., 2001; Yang

et al., 2001). Time-lapse imaging *in vivo* shows that some AChR aggregates are organized by the nerves, whereas others may be recognized by the nerve and incorporated into the synapse (Flanagan-Steet et al., 2005; Panzer et al., 2006). Partially redundant control by intrinsic and extrinsic patterning may help ensure that all muscle fibers end up with proper innervation (Kummer et al., 2006; Lin et al., 2008).

A combination of transsynaptic and cell-autonomous factors may also be responsible for postnatal maturation of the NMJ. During the first 2 wk after birth, the initially ovoid postsynaptic plaque of AChRs is transformed into a complex pretzel-shaped array of branches (Slater, 1982; Desaki and Uehara, 1987; Marques et al., 2000; Sanes and Lichtman, 2001). This process, which mirrors the postnatal branching of nerve terminals, was long assumed to reflect transsynaptic signaling because it fails to occur in neonatally denervated muscles, and because AChR aggregates in cultured myotubes generally remain plaque-shaped.

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Abbreviations used in this paper: AChR, acetylcholine receptor; Bcam, basal cell adhesion molecule/Lutheran blood group antigen; BTX, α -bungarotoxin; LG, laminin globular; MuSK, muscle-specific kinase; NMJ, neuromuscular junction.

The online version of this article contains supplemental material.

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Recently, however, we found that the plaque-to-pretzel transition can occur to a remarkable extent in myotubes cultured aneurally (Kummer et al., 2004). Thus, mechanisms must exist that coordinate the branching of pre- and postsynaptic structures as synapses mature.

How can such coordination be achieved? One attractive hypothesis is that some components of the synaptic cleft influence both pre- and postsynaptic differentiation. In support of this idea, we provide evidence here that laminins, muscle-derived synaptic organizers that promote presynaptic differentiation by an intercellular mechanism, also promote postsynaptic maturation by an autocrine mechanism. Laminins, which are heterotrimers of α , β , and γ subunits, are components of the basal lamina that ensheathes muscle fibers. Most of the basal lamina is rich in laminin 211 ($=\alpha 2$, $\beta 1$, $\gamma 1$; see Aumailley et al., 2005 for laminin nomenclature), and contains the $\beta 1$ subunit. In contrast, the small fraction of the basal lamina that occupies the synaptic cleft at the NMJ bears little if any $\beta 1$ but is rich in laminins 221, 421, and 521, all of which contain the $\beta 2$ subunit (Patton et al., 1997). Laminin $\beta 2$ promotes presynaptic differentiation, in part by binding to voltage-gated calcium channels in the presynaptic membrane (Noakes et al., 1995; Son et al., 1999; Nishimune, et al., 2004).

In attempting to determine roles of the individual laminin α chains associated with synaptic $\beta 2$ ($\alpha 2$, $\alpha 4$, and $\alpha 5$ in laminins 221, 421, and 521, respectively), we found ultrastructural defects in alignment of pre- and postsynaptic specializations in mice lacking laminin $\alpha 4$ (Patton et al., 2001). Lack of laminin $\alpha 2$ also leads to modest neuromuscular defects, but these may be secondary to the muscular dystrophy that results from loss of laminin 211 (Law et al., 1983; Desaki et al., 1995; Patton et al., 2001). Roles of laminin $\alpha 5$ have been difficult to analyze because mice lacking laminin $\alpha 5$ (*Lama5*^{-/-}) die at late embryonic stages because of multiple developmental defects, including exencephaly, syndactyly, and dysmorphogenesis of the placenta (Miner et al., 1998). Accordingly, we reexamined this issue using a conditional mutant (Nguyen et al., 2005) in which we could selectively remove laminin $\alpha 5$ from muscle. Postsynaptic maturation was delayed in mice lacking laminin $\alpha 5$ in muscle and arrested in double mutants lacking laminins $\alpha 4$ and $\alpha 5$. Further analysis, both in vivo and in vitro, provided evidence that laminin acts directly on myotubes to promote postsynaptic maturation and that this autocrine effect is mediated in large part by the transmembrane laminin receptor dystroglycan. Together, these results suggest that synaptic laminins act through distinct receptors as both retrograde organizers of presynaptic maturation and autocrine organizers of postsynaptic maturation. This dual role provides an elegant way to coordinate pre- and postsynaptic growth and differentiation as the synapse matures.

Results

Muscle-specific deletion of laminin $\alpha 5$

In initial studies, we examined muscles from targeted laminin $\alpha 5$ null mutant mice (*Lama5*^{-/-}; Miner et al., 1998). NMJs formed in these mutants, with vesicle-rich nerve terminals apposed to postsynaptic AChR aggregates (Biernan, J., L. Knittel, D. Yang, Y.S. Tarumi, J.H. Miner, J.R. Sanes, and B.L. Patton. 2003.

Society for Neuroscience 33rd Annual Meeting. Abstr. 898.7). This analysis was limited to embryonic stages, however, because *Lama5*^{-/-} mutants usually die before embryonic day 17 (Miner et al., 1998). To assess roles of this subunit in later stages of neuromuscular synaptogenesis, we used a conditional allele in which exons 15–21 of the *Lama5* gene were flanked by loxP sites (*Lama5*^{loxP}; Nguyen et al., 2005). Cre-mediated recombination of the loxP sites excises these exons, resulting in a frame-shifted laminin $\alpha 5$ mRNA and a null allele. We mated the conditional mutants to transgenic mice in which regulatory sequences from the human skeletal actin gene drive muscle-specific expression of Cre recombinase (*HSA-Cre*; Schwander et al., 2003). We refer to the resulting *Lama5*^{loxP/loxP}; *HSA-Cre* mutants as *Lama5*^{M/M} mice. *Lama5*^{M/M} mice were externally normal, fertile, and lived for at least 2 yr.

In wild-type mice, laminin $\alpha 5$ is present throughout the basal lamina of embryonic myotubes. Over the first three postnatal weeks, laminin $\alpha 5$ is lost from extrasynaptic basal lamina and becomes concentrated in synaptic basal lamina (Patton et al., 1997; Fig. 1 E). In *Lama5*^{M/M} muscle, laminin $\alpha 5$ was barely detectable in extrasynaptic basal lamina at postnatal day 0; it was undetectable at 75% of NMJs at P0 and at 95% of NMJs by P10 (Fig. 1 A and not depicted). The low levels of laminin $\alpha 5$ present at birth may result from slow degradation of laminins inserted before gene deletion (see Cohn et al., 2002). In any case, the absence of laminin $\alpha 5$ from NMJs at later stages indicates that synaptic deposits of this subunit in postnatal muscle are contributed solely by the muscle and not by motor neurons or Schwann cells. Quantitative analysis of immunofluorescence indicated that levels of laminins $\alpha 4$ and $\beta 2$ at the NMJ were unaffected by the absence of laminin $\alpha 5$ (Fig. 1 A and not depicted).

Delayed synaptic maturation in the absence of laminin $\alpha 5$

At birth, postsynaptic AChR clusters at normal NMJs are ovoid plaques; during the first three postnatal weeks, they are transformed to perforated and then C-shaped aggregates, and finally to pretzel-shaped branched arrays (Slater, 1982; Desaki and Uehara, 1987; Marques et al., 2000; Kummer et al., 2004). To examine whether this process requires laminin $\alpha 5$, we stained longitudinal sections of muscles from *Lama5*^{M/M} mice and littermate controls with fluorescently labeled α -bungarotoxin (BTX), which binds specifically to AChRs. We detected no difference between *Lama5*^{M/M} and control muscles during the first few postnatal days or in adulthood (Fig. 1 C). Examination of intermediate time points showed, however, that the topological transformation of AChR clusters was delayed in *Lama5*^{M/M} mice (Fig. 1 B). In sternomastoid muscle, for example, maturation was delayed by >1 wk (Fig. 1 C). Similar results were obtained in diaphragm (unpublished data). Thus, muscle-derived laminin $\alpha 5$ promotes topological maturation of the postsynaptic membrane at the NMJ.

To assess roles of laminin $\alpha 5$ in presynaptic differentiation, we stained *Lama5*^{M/M} muscle with antibodies to a synaptic vesicle protein (SV2) and an axonal antigen (neurofilament). In control animals, motor nerve terminals were precisely apposed to AChR-rich postsynaptic sites at >80% of NMJs at P21 and

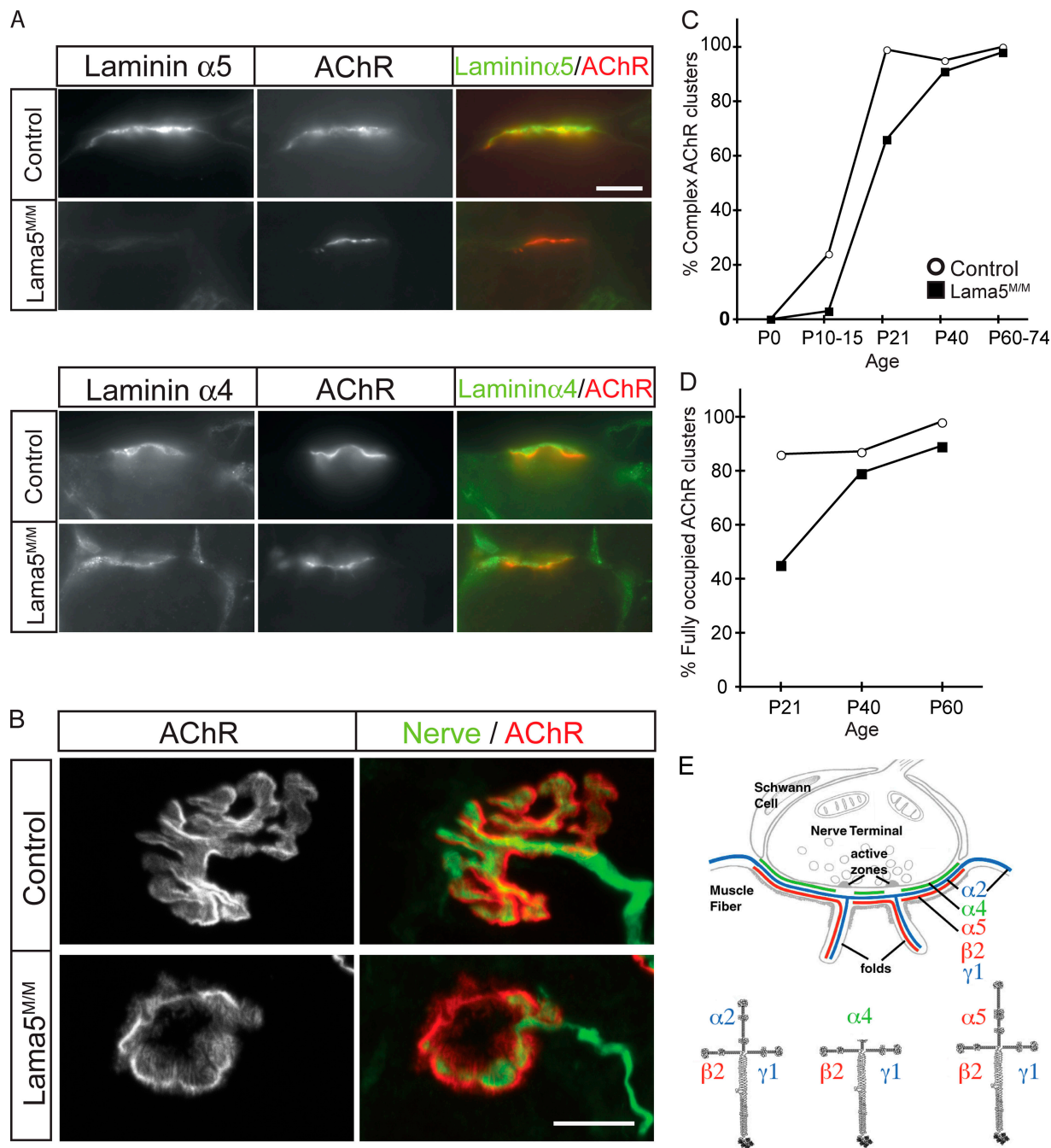


Figure 1. Muscle-specific deletion of laminin $\alpha 5$ leads to delayed neuromuscular synapse maturation. (A) Laminin $\alpha 5$ protein was undetectable at NMJs by P21 in *Lama5^{M/M}* mice, but laminin $\alpha 4$ levels were unaltered. Muscle sections were stained with laminin antibodies (green) and Alexa 594-BTX to label AChRs (red). (B) En face views of NMJs in P21 control and *Lama5^{M/M}* mice stained with antibodies to neurofilaments and SV2 (nerve, green), and Alexa 594-BTX (AChR, red). Motor nerve terminals fully occupied complex AChR clusters in controls. In *Lama5^{M/M}* muscle, many AChR clusters had a simple morphology and were partially innervated. (C and D) Quantification of topological maturation of AChR clusters (C) and completeness of innervation of AChR clusters (D) in control (open circles) and *Lama5^{M/M}* (closed squares) sternomastoid muscle. Counts are from two animals per age, >40 NMJs per animal, with the exception of controls at P21 and P40, which are from one animal. (E) Laminin chains present in synaptic BL (adapted from Patton et al., 1997). Bars, 10 μ m.

>90% of NMJs by P60 (Fig. 1, B and D). In *Lama5^{M/M}* mice, in contrast, motor nerves only partially covered the postsynaptic membrane of many NMJs between P21 and P40 (Fig. 1, B and D). At later times, however, apposition was achieved in both sternomastoid and diaphragm muscles (Fig. 1 D and not depicted). Thus, pre- as well as postsynaptic differentiation is delayed in *Lama5^{M/M}* mutants.

A chimeric laminin α chain rescues pre- but not postsynaptic defects in laminin $\alpha 5$ mutants

As synaptic laminins have been implicated in presynaptic differentiation (Noakes et al., 1995; Patton et al., 1997, 1998, 2001; Nishimune et al., 2004), we considered the possibility that defects in postsynaptic maturation in *Lama5^{M/M}* muscle

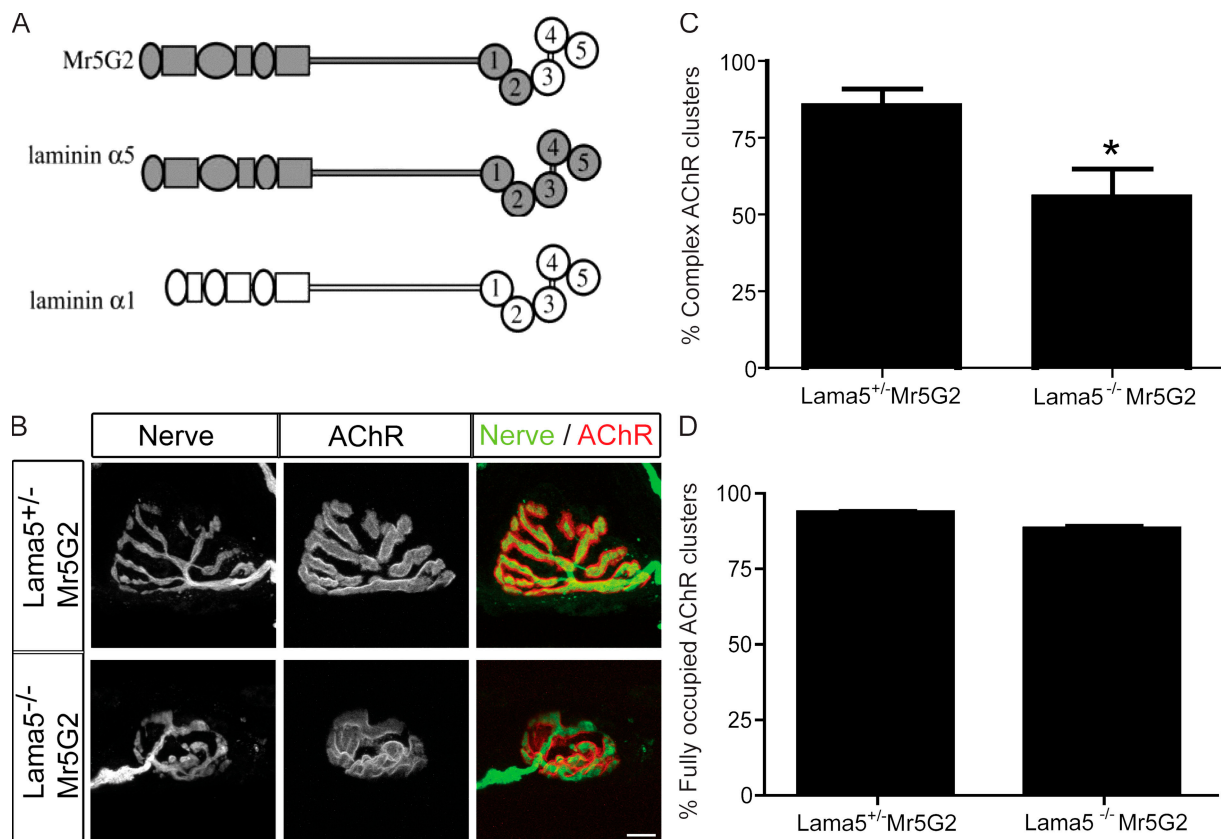


Figure 2. Chimeric laminin $\alpha 5$ chain rescues the pre- but not postsynaptic phenotype in *Lama5* null mice. (A) Structure of the *Mr5G2* transgene, in which the C-terminal three LG domains of laminin $\alpha 5$ are replaced by the corresponding domains of laminin $\alpha 1$. (B) NMJs from control (*Lama5*^{+/-}*Mr5G2*) and *Lama5*^{-/-}*Mr5G2* sternomastoid at P21 stained as in Fig. 1 B. AChR clusters in the mutant are small and immature but fully occupied by motor neuron terminals. Bar, 10 μ m. (C and D) Quantification of topological maturation of AChR clusters (C) and completeness of innervation of AChR clusters (D) in control (*Lama5*^{+/-}*Mr5G2*) and *Lama5*^{-/-}*Mr5G2* muscles. Counts are from sternomastoid and diaphragm muscles of two animals per genotype, >190 NMJ per animal. *, significant by unpaired *t* test, *P* < 0.05.

might be secondary consequences of the presynaptic defects. In an attempt to differentially rescue pre- and postsynaptic phenotypes, we used transgenic mice that ubiquitously express a chimeric laminin subunit in which the C-terminal ~540 amino acids of $\alpha 5$ are replaced by the homologous sequences from laminin $\alpha 1$ (Kikkawa et al., 2002; Fig. 2 A). This region comprises the terminal three of five laminin globular (LG) domains present in all laminin α chains. LG domains contain binding sites for several transmembrane proteins that bind laminin; some bind differentially to the LG domains in the $\alpha 1$ and $\alpha 5$ chains (Kikkawa and Miner, 2005; Nishiuchi et al., 2006; Kikkawa et al., 2007). The chimera transgene, called *Mr5G2*, rescues the embryonic lethality of *Lama5*^{-/-} null mutants: *Lama5*^{-/-}*Mr5G2* mice are outwardly normal and live for up to several months before succumbing to kidney disease (Kikkawa and Miner, 2006).

We analyzed *Lama5*^{-/-}*Mr5G2* mice at P21, when both pre- and postsynaptic differentiation are impaired in *Lama5*^{M/M} mice. At this age, maturation of the postsynaptic apparatus was as incomplete in *Lama5*^{-/-}*Mr5G2* muscle as in *Lama5*^{M/M} muscle (Fig. 2, B and C). In contrast, whereas innervation was incomplete at this age in *Lama5*^{M/M} muscle, motor axons completely occupied AChR-rich postsynaptic sites in *Lama5*^{-/-}*Mr5G2* muscle (Fig. 2, B and D). Thus, the *Mr5G2* chimera pro-

motes presynaptic maturation effectively but postsynaptic maturation poorly.

We draw three conclusions from the observation that postsynaptic defects persist despite rescue of presynaptic defects by the *Mr5G2* transgene. First, postsynaptic defects may result from a direct action of laminin on the postsynaptic apparatus. Second, receptors that mediate the postsynaptic effects of laminin interact differentially with the final three LG domains of laminins $\alpha 1$ and $\alpha 5$. Finally, sites shared by laminins $\alpha 1$ and $\alpha 5$ interact with nerve terminals to promote complete coverage of postsynaptic sites.

Arrested synaptic maturation in muscles lacking laminins $\alpha 4$ and $\alpha 5$

Because synaptic defects in *Lama5*^{M/M} mice were transient, we considered the possibility that roles of laminin $\alpha 5$ were partially redundant with or compensated by another synaptic cleft component. A likely candidate was laminin $\alpha 4$, the other laminin α chain selectively associated with synaptic basal lamina. Laminin $\alpha 4$ remained concentrated at synapses in *Lama5*^{M/M} muscle (Fig. 1 A). Targeted mutants lacking laminin $\alpha 4$ (*Lama4*^{-/-}) are healthy and fertile (Patton et al., 2001), so we were able to test this possibility by generating *HSA-Cre*;*Lama4*^{-/-};*Lama5*^{loxP/loxP} (*Lama4*^{-/-};*Lama5*^{M/M}) double mutants.

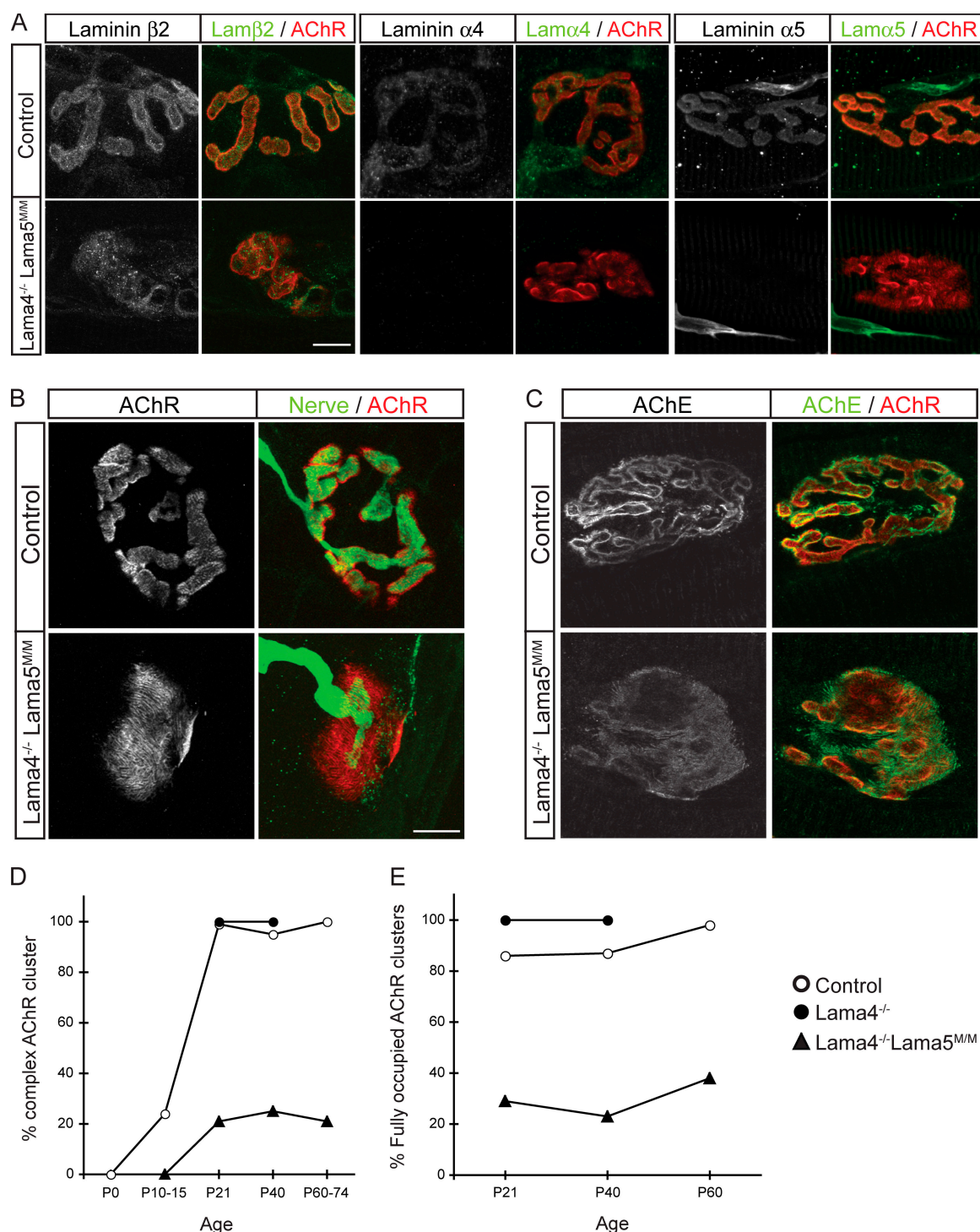
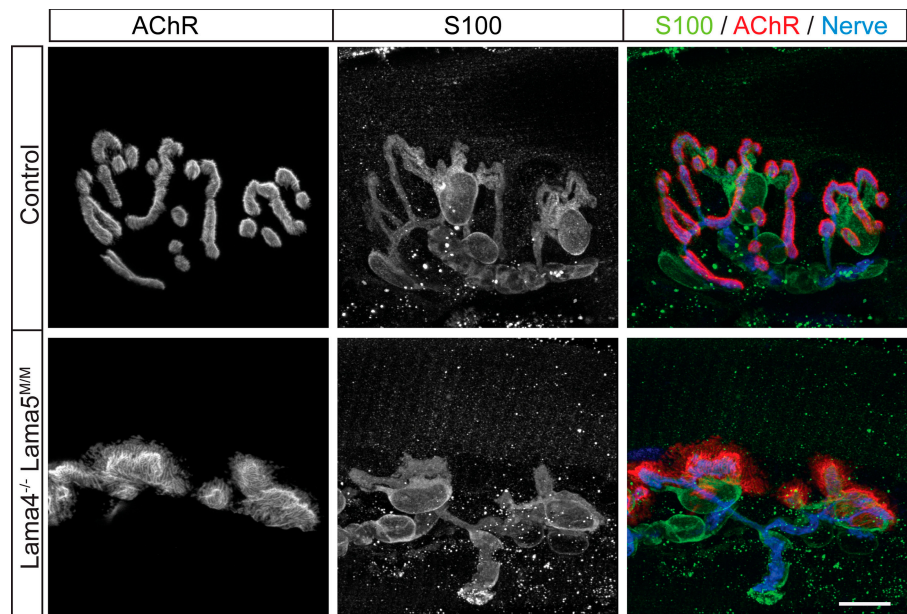


Figure 3. Arrest of postsynaptic maturation in *Lama4*^{-/-};*Lama5*^{M/M} mice. (A) NMJs from *Lama4*^{-/-};*Lama5*^{M/M} mice at P36 lack laminins $\alpha 4$ and $\alpha 5$ but retain laminin $\beta 2$. (B) Most postsynaptic sites retained a simple plaque-like topology and remained only partially occupied by nerve terminals in *Lama4*^{-/-};*Lama5*^{M/M} mice at P60. (C) NMJs from *Lama4*^{-/-};*Lama5*^{M/M} mice at P36 retained acetylcholinesterase (AChE). Bars, 10 μ m. (D and E) Quantification of topological maturation of AChR clusters (D) and completeness of innervation of AChR clusters (E) in control (open circles), *Lama4*^{-/-} (closed circles), and *Lama4*^{-/-};*Lama5*^{M/M} mice (closed triangles). Counts are from two animals per age, >40 NMJs per animal.

NMJs in *Lama4*^{-/-} mice are smaller than those in controls and exhibit ultrastructural defects in alignment of specializations in nerve terminals with those in the postsynaptic membrane (Patton et al., 2001). However, maturation of the postsynaptic membrane to a branched array is not perturbed in *Lama4*^{-/-}

mice (Fig. 3 D). *Lama4*^{-/-};*Lama5*^{M/M} double mutants were smaller and weaker than either single mutant (Fig. S1, available at <http://www.jcb.org/cgi/content/full/jcb.200805095/DC1>), and most died around 3 mo of age. During the first two postnatal weeks, NMJs in *Lama4*^{-/-};*Lama5*^{M/M} mice resembled those in

Figure 4. Schwann cells colocalize with motor nerve terminals in *Lama4*^{-/-};*Lama5*^{M/M} NMJ. En face view of NMJs from *Lama4*^{-/-};*Lama5*^{M/M} and control mice were stained with antibodies against Schwann cells (S100, green), nerve terminals (neurofilament + SV2, blue), and AChRs (BTX, red). Schwann cells colocalized with the nerve terminal but did not cover the unoccupied AChR clusters in the mutant. Bar, 10 μ m.



Lama5^{M/M} mice. Subsequently, however, although pre- and post-synaptic maturation were delayed in *Lama5*^{M/M} mice and unaffected in *Lama4*^{-/-} mice, they were arrested in *Lama4*^{-/-};*Lama5*^{M/M} mutants. Similar results were seen in sternomastoid, diaphragm, and tibialis anterior muscles (Fig. 3, B, D, and E; Fig. S2; and not depicted). Arrest of the topological transformation from plaque-shaped to branched postsynaptic apparatus was evident using markers of the synaptic cleft (agrin, acetylcholinesterase, and glycoconjugates recognized by the VVA-B4 lectin; Fig. 3 C and not depicted) as well as AChRs.

To ask whether the apparent arrest in postsynaptic maturation resulted from ongoing muscle degeneration and regeneration, we counted central nuclei, a marker of regenerating muscle fibers that persists for several months (Schmalbruch, 1976). The number of central nuclei did not differ significantly between double mutants and controls (unpublished data).

Schwann cells are the third cell of the NMJ. They respond to laminins, and defects in synaptic Schwann cells are evident in mice lacking laminin β 2 (Patton et al., 1998). We therefore assessed the distribution of terminal Schwann cells in *Lama4*^{-/-};*Lama5*^{M/M} mice by staining muscles with antibodies to the Schwann cell marker S-100 β . In mutants as in controls, Schwann cells capped nerve terminals, and only portions of postsynaptic membrane devoid of nerve terminals lacked Schwann cells (Fig. 4). These results suggest that the abnormal distribution of Schwann cells in *Lama4*^{-/-};*Lama5*^{M/M} mutants is secondary to presynaptic defects.

Previous studies have revealed a prominent role for laminin β 2 in maturation of nerve terminals and synaptic Schwann cells (Noakes et al., 1995; Patton et al., 1998; Nishimune et al., 2004). We therefore considered the possibility that loss of laminins α 4 and α 5 might impair synaptic maturation indirectly by leading to loss of laminin β 2. However, laminin β 2 immunoreactivity persisted at synaptic sites (Fig. 3 A). Although levels of laminin β 2 immunoreactivity varied among NMJs, defects were seen in laminin β 2-rich as well as in laminin β 2-poor NMJs of *Lama4*^{-/-};*Lama5*^{M/M} mutants.

Direct effect of laminins α 4 and α 5 on myotubes

Analysis of *Lama5*^{-/-};*Mr5G2* mice (Fig. 2) suggested that synaptic laminins exert a direct effect on topological maturation of AChR clusters. For a direct test of this idea, we cultured muscle cells from wild-type and mutant mice in the absence of neurons. We dissociated myoblasts from control and *Lama4*^{-/-};*Lama5*^{M/M} mice, cultured them on substrates coated with laminin 111, and allowed them to fuse into myotubes. Under these conditions, complex, branched AChR clusters form on the myotube surface that contacts the substrate, and laminin α 5 concentrates in apposition to the AChR clusters (Kummer et al., 2004; Fig. 5, A and B). Clusters also formed on *Lama4*^{-/-};*Lama5*^{M/M} myotubes, but the fraction of clusters with a complex morphology after 8 d in vitro was $\sim$ 60% lower in *Lama4*^{-/-};*Lama5*^{M/M} myotubes than in controls ($\sim$ 20% vs. $\sim$ 50%; Fig. 5, B and C). Thus, *Lama4*^{-/-};*Lama5*^{M/M} myotubes are deficient in their ability to form branched arrays of AChRs. To determine whether these results depended on the presence of a distinct laminin (laminin 111) on the substrate, we also cultured control and *Lama4*^{-/-};*Lama5*^{M/M} muscle cells on fibronectin. Again, the proportion of complex AChR clusters was lower on *Lama4*^{-/-};*Lama5*^{M/M} myotubes than on control (Fig. 5 D).

We also prepared cultures from *Lama5*^{M/M}, *Lama4*^{-/-}, and Laminin β 2 (*Lamb2*^{-/-}) single mutant mice. Myotubes from *Lama4*^{-/-} and *Lamb2*^{-/-} mice exhibited the same proportion of complex receptor clusters as did controls (Fig. 5 C and D). In contrast, myotubes from *Lama5*^{M/M} mice showed fewer complex receptor clusters than controls; this difference was not statistically significant in myotubes grown on laminin 111 but was significant in myotubes grown on fibronectin (Figs. 5, C and D). Collectively with observations in vivo, these results suggest that laminin α 5 plays a dominant role in postsynaptic maturation but that laminin α 4 can partially compensate for its loss. The deficiency in topological maturation was not an indirect consequence of impaired ability to form clusters because soluble

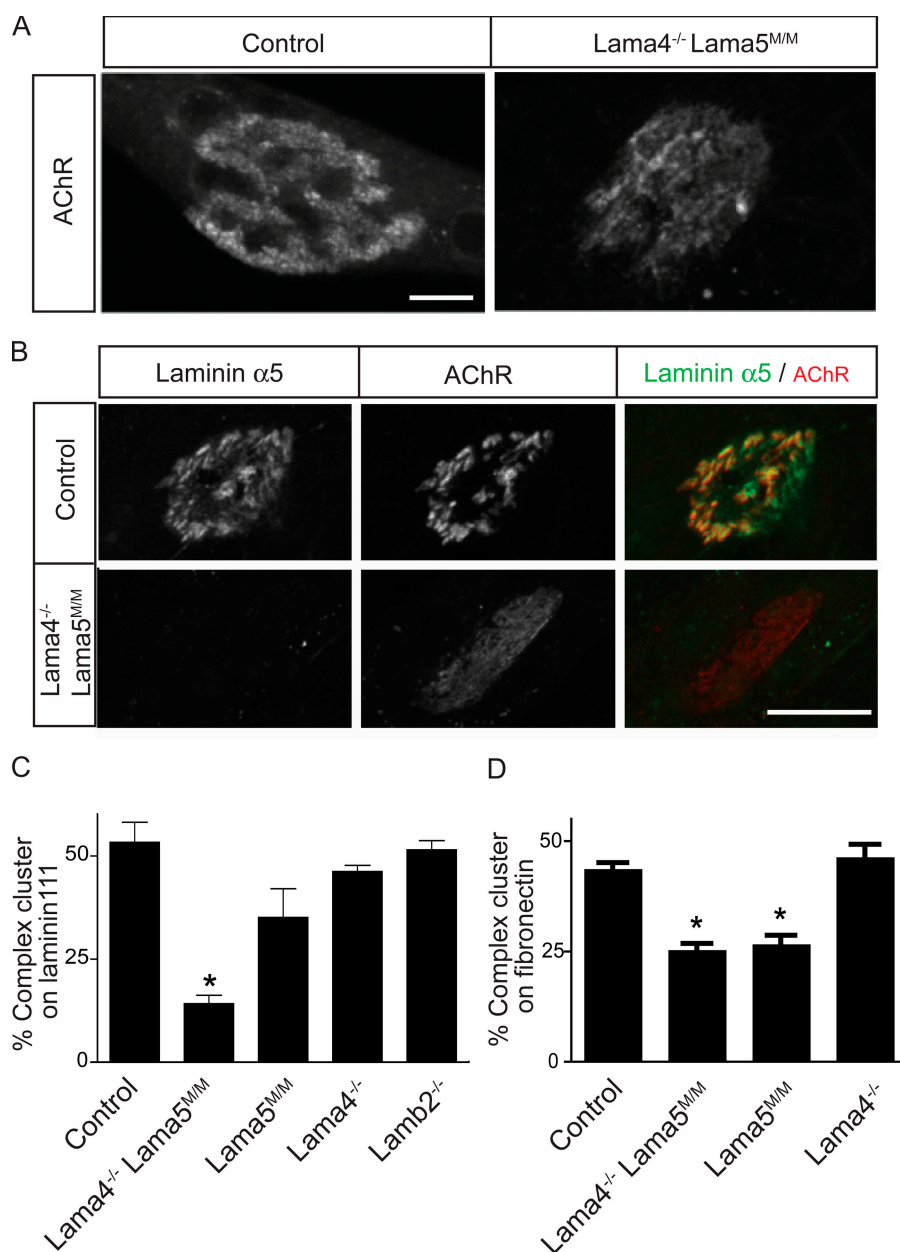


Figure 5. Laminins $\alpha 4$ and $\alpha 5$ exert an auto-crine effect on postsynaptic maturation. (A) Myotubes from control and *Lama4*^{-/-};*Lama5*^{M/M} mice were cultured on laminin 111 and stained for AChRs with Alexa 594-BTX. Micrographs show a complex AChR cluster on a control myotube and a simple AChR plaque on a *Lama4*^{-/-};*Lama5*^{M/M} myotube. (B) Laminin $\alpha 5$ is associated with AChR clusters in control myotubes but was absent from clusters in *Lama4*^{-/-};*Lama5*^{M/M}. Bars, 10 μ m. (C and D) Quantification of AChR aggregate topology on myotubes from control, *Lama4*^{-/-};*Lama5*^{M/M}, *Lama5*^{M/M}, *Lama4*^{-/-}, and *Lamb2*^{-/-} myotubes cultured on laminin 111 (C) or fibronectin (D). Myotubes from *Lama4*^{-/-};*Lama5*^{M/M} and *Lama5*^{M/M} mice form fewer complex AChR clusters on both substrates than do control, *Lama4*^{-/-}, or *Lamb2*^{-/-} myotubes. For each experiment, at least 150 AChR clusters were examined. Counts show mean $\pm$ SEM from three animals per genotype. *, significant by one-way analysis of variance, $P < 0.0001$.

agrin induced similar numbers of AChR clusters on primary myotubes prepared from control, *Lama5*^{M/M}, or *Lama4*^{-/-};*Lama5*^{M/M} mice (Fig. S3, available at <http://www.jcb.org/cgi/content/full/jcb.200805095/DC1>). These results indicate that laminins $\alpha 4$ and $\alpha 5$ signal directly to myotubes to induce topological maturation of AChR clusters.

Effect of laminin on postsynaptic localization of laminin receptors

Analysis of the chimeric laminin described above (*Mr5G2*; Fig. 2) suggested that sites in globular domains LG3-5 of laminin $\alpha 5$ interact with receptors on muscle fibers. Three laminin-binding proteins have been shown to bind to this region: dystroglycan, $\beta 1$ integrins, and basal cell adhesion molecule/Lutheran blood group antigen (Bcam), an immunoglobulin superfamily adhesion mol-

ecule (Belkin and Stepp, 2000; Timpl et al., 2000; Kikkawa and Miner, 2005; Suzuki et al., 2005; Barresi and Campbell, 2006; Nishiuchi et al., 2006). All three receptors are expressed by muscle and present in postsynaptic regions of the muscle fiber membrane (Fig. 6; Matsumura et al., 1992; Martin et al., 1996). They are therefore candidate mediators of the postsynaptic effects of laminins. In several cases, laminins have been shown to promote aggregation of their receptors (Colognato et al., 1999; Moulson et al., 2001; Marangi et al., 2002; Smirnov et al., 2002). As a first test of whether synaptic laminins interact with these receptors, we therefore asked whether their synaptic localization was perturbed in *Lama5*^{M/M} or *Lama4*^{-/-};*Lama5*^{M/M} mutants.

Dystroglycan is present throughout the muscle fiber surface, but it is present at higher levels in the postsynaptic membrane than in extrasynaptic regions (Matsumura et al., 1992;

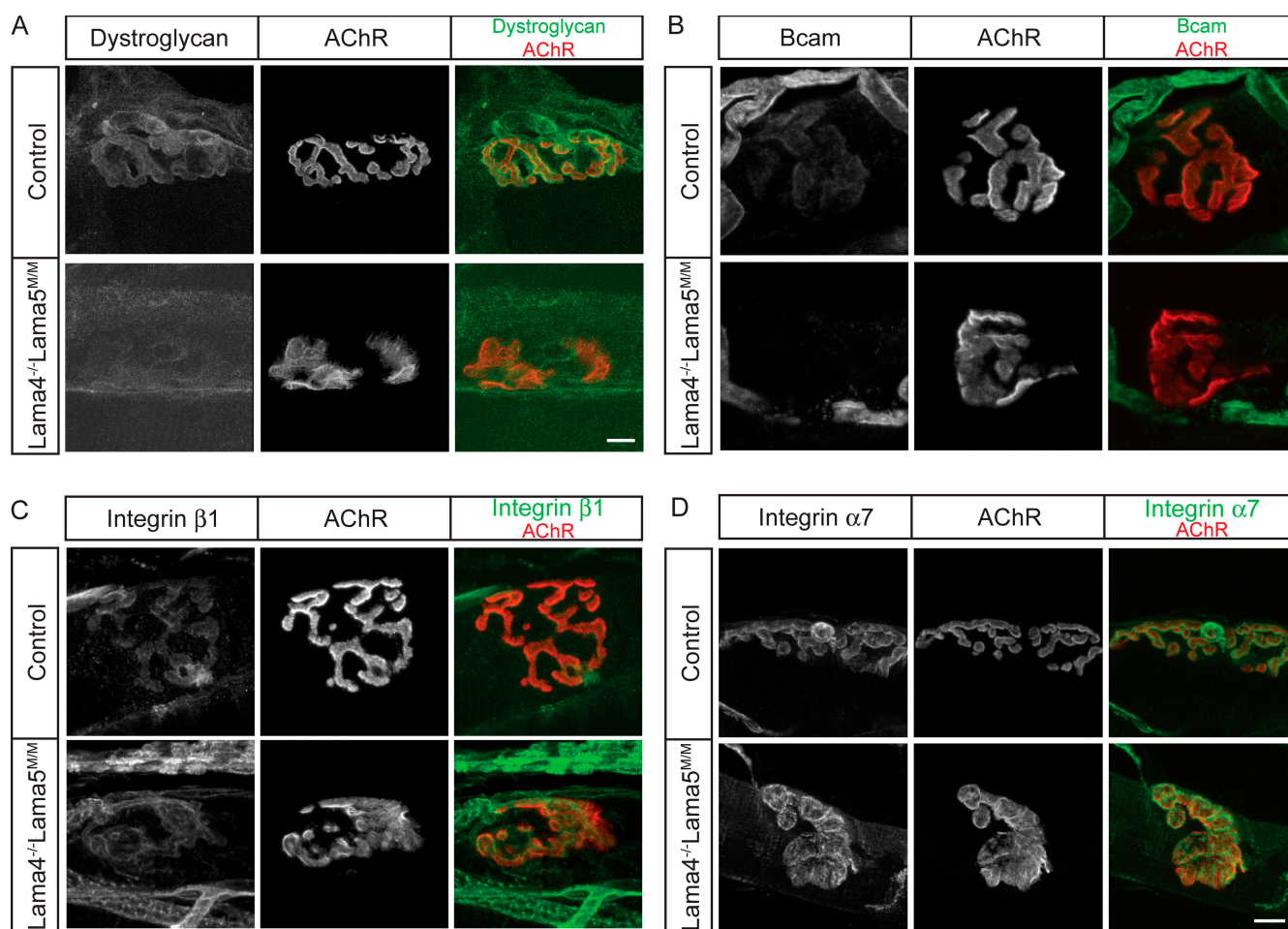


Figure 6. Distribution of laminin receptors at NMJs of *Lama4*^{-/-};*Lama5*^{M/M} mice. (A and B) Dystroglycan (A) and Bcam (B) are present at higher levels in synaptic rather than extrasynaptic regions of muscle fibers in control mice. Synaptic levels of both antigens were decreased *Lama4*^{-/-};*Lama5*^{M/M} muscle. (C and D) Integrin β1 (C) and integrin α7 (D) are concentrated at synaptic regions of both control and *Lama4*^{-/-};*Lama5*^{M/M} muscle fibers. Longitudinal sections of sternomastoid at P60–79 muscle were stained with antibodies to laminin receptors (green) and AChRs (red). Bars, 10 μm.

Cohen et al., 1995). The synaptic concentration of dystroglycan persisted in adult *Lama5*^{M/M} and *Lama4*^{-/-} muscle but was greatly reduced in *Lama4*^{-/-};*Lama5*^{M/M} muscle (Fig. 6 A and not depicted). Extrasynaptic levels of dystroglycan immunoreactivity were not detectably different in *Lama5*^{M/M} or *Lama4*^{-/-};*Lama5*^{M/M} mice than in littermate controls.

Bcam is present in the membrane of embryonic myotubes (Moulson et al., 2001). We found that Bcam levels decline in extrasynaptic regions of muscle fibers postnatally but that Bcam immunoreactivity persists at synaptic sites into adulthood (Fig. 6 B). In contrast, Bcam was barely detectable in the synaptic membranes of either *Lama5*^{M/M} or *Lama4*^{-/-};*Lama5*^{M/M} mice (Fig. 6 B and not depicted).

Integrins are α/β heterodimers that bind a variety of matrix molecules. Several integrins are receptors for laminins, including α3β1, α6β1, and α7β1. We stained sections of control, *Lama4*^{-/-}, *Lama5*^{M/M}, and *Lama4*^{-/-};*Lama5*^{M/M} mice for the common integrin β1 subunit but saw no difference between controls and any of the mutants; in all cases, integrin β1 was present throughout the muscle fiber membrane, with higher levels in synaptic rather than extrasynaptic regions (Fig. 6 C and

not depicted). We also stained muscle for integrin α7, which is known to be concentrated in the postsynaptic membrane (Martin et al., 1996; Burkin and Kaufman, 1999). Again, no differences in level or localization were observed between controls and any of these mutants (Fig. 6 D and not depicted).

These results are consistent with the idea that interactions of dystroglycan and Bcam with laminin α5, either in conjunction with laminin α4 (dystroglycan) or alone (Bcam), promote aggregation of these receptors in the postsynaptic membrane. Alternatively, however, the absence of specific laminins might influence presynaptic structures, which in turn might be responsible for localizing dystroglycan and Bcam. To distinguish these possibilities, we used the culture system illustrated in Fig. 5. High levels of both dystroglycan and Bcam were associated with AChR clusters in control myotubes (Fig. 7, A and B). In myotubes from *Lama4*^{-/-};*Lama5*^{M/M} mice, in contrast, levels of dystroglycan were significantly decreased, and levels of Bcam were dramatically decreased at large AChR clusters whether laminin 111 or fibronectin were used for culture substrate (Fig. 7, A–D).

Further analysis of these cultures revealed two additional features of *Lama4*^{-/-};*Lama5*^{M/M} myotubes. First, low levels of

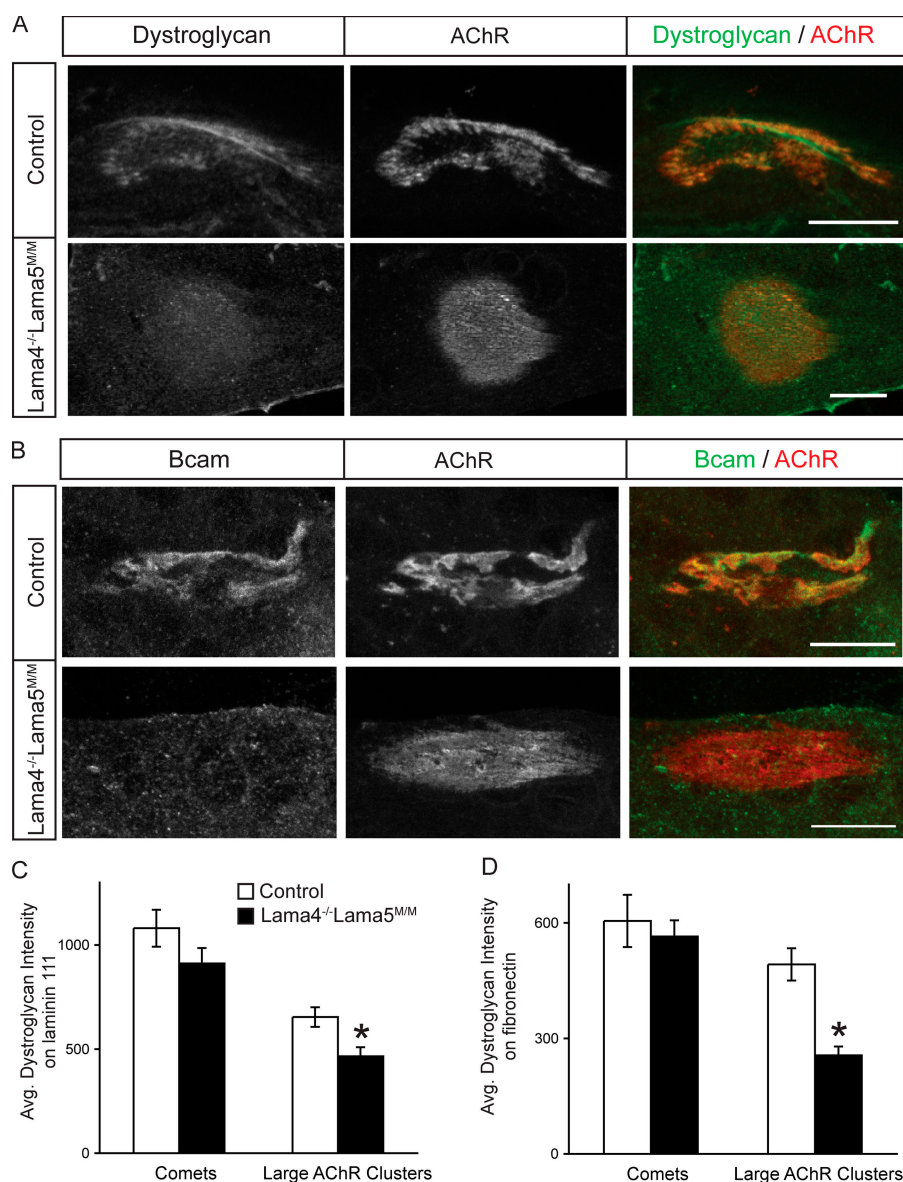


Figure 7. Decreased dystroglycan and Bcam at AChR clusters lacking laminins $\alpha 4$ and $\alpha 5$. (A and B) Control and *Lama4^{-/-}Lama5^{MM}* cultures were stained for AChR, dystroglycan (A) and Bcam (B). Levels of both antigens are decreased at clusters on mutant myotubes. Bars, 10 μ m. (C and D) Dystroglycan relative fluorescence intensity in AChR clusters from control and *Lama4^{-/-}Lama5^{MM}* myotubes cultured on laminin 111 (C) or fibronectin (D). Counts show mean $\pm$ SEM from at least three independent cultures, >75 AChR clusters per culture. *, significant by unpaired *t* test, *P* < 0.002.

laminin $\alpha 5$ persisted at AChR-rich clusters in some mutant myotubes. The residual laminin might have been synthesized by muscle cells before Cre-mediated excision of the *Lama5* gene. Second, although levels of dystroglycan were decreased at plaque-shaped and branched AChR clusters in *Lama4^{-/-}Lama5^{MM}* myotubes, they remained high in small linear or comet-shaped AChR clusters resembling those formed spontaneously or after application of agrin to myotubes cultured on gelatin (Fig. S4, available at <http://www.jcb.org/cgi/content/full/jcb.200805095/DC1>). In contrast, levels of Bcam were decreased at all AChR clusters in mutants. This result raises the possibility that initial association of dystroglycan with AChR clusters is laminin independent, whereas maintenance of that association is laminin dependent.

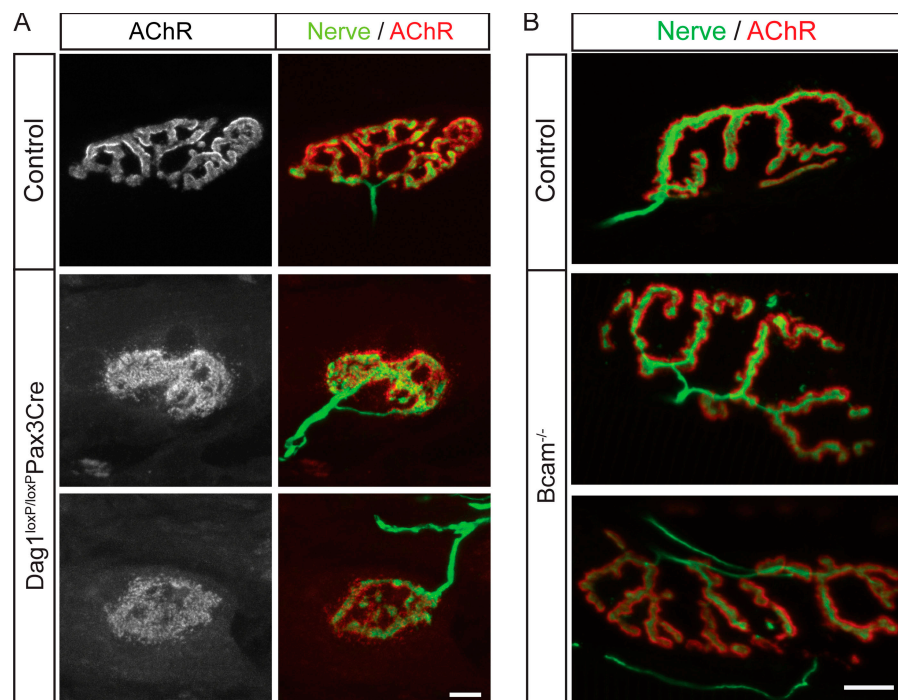
Dystroglycan but not Bcam promotes postsynaptic maturation

If laminin-dependent aggregation of dystroglycan or Bcam promotes postsynaptic maturation, one would expect that synapses

would remain immature in the absence of the critical laminin-binding protein. To test this idea, we analyzed mice lacking dystroglycan or Bcam.

Dystroglycan null mice (*Dag1^{-/-}*) die early in gestation because of disruption of extraembryonic membranes (Williamson et al., 1997). We therefore used a conditional *Dag1* allele (*Dag1^{loxP/loxP}*; Cohn et al., 2002). These mice were mated to transgenic mice that express Cre recombinase under the control of regulatory elements from the *Pax3* gene (*Pax3-Cre*; Li et al., 2000). *Dag1^{loxP/loxP};Pax3-Cre* mice are viable, but dystroglycan is efficiently eliminated from a subset of skeletal muscles, including the tibialis anterior of the hind limb (unpublished data). In tibialis muscle of P24 *Dag1^{loxP/loxP};Pax3-Cre* mice, AChR clusters were complex at only 3% of NMJs (Fig. 8 A). The post-synaptic membrane was complex at 97% of control NMJs by this age. Counts of central nuclei (see above) showed that ~11% of muscle fibers in the tibialis anterior of *Dag1^{loxP/loxP};Pax3-Cre* had undergone at least one cycle of degeneration and regeneration

Figure 8. **Dystroglycan but not Bcam is required for postsynaptic maturation.** (A) AChR clusters in *Dag1^{loxP/loxP};Pax3-Cre* mice were plaque-like in topology. (B) NMJs in *Bcam^{-/-}* mice are indistinguishable in topology from those in littermate controls. Longitudinal sections of adult tibialis muscles were stained with antibodies to neurofilaments and SV2 (nerve, green), and Alexa 594-BTX (AChR, red). Bars, 10 μ m.



by P24 (unpublished data). As postsynaptic maturation was impaired in 97% of muscle fibers, we conclude that the synaptic defects were not an indirect consequence of muscle damage. These results are consistent with those obtained previously in chimeric mice and cultured myotubes (see Discussion). Thus, postsynaptic maturation is arrested in *Dag1^{loxP/loxP};Pax3-Cre* mice, as it is in *Lama4^{-/-};Lama5^{MM}* mice. In contrast, nerve terminals fully covered postsynaptic sites in *Dag1^{loxP/loxP};Pax3-Cre* mice, whereas, as shown in Fig. 3, innervation was incomplete in *Lama4^{-/-};Lama5^{MM}* muscle. These results support the idea that dystroglycan mediates the autocrine effects of laminin on postsynaptic maturation.

To assess the role of Bcam at the NMJ, we generated targeted null mutants (see Materials and methods). *Bcam^{-/-}* mice were viable, fertile and overtly healthy (unpublished data), similar to those described recently (Rahuel et al., 2008). We examined NMJs in the sternomastoid, diaphragm, and tibialis anterior muscles of *Bcam^{-/-}* mice at both P21 and in adulthood (P49 and P330). In all muscles at both ages, the structure and maturity of NMJs in mutants was indistinguishable from those in littermate controls (Fig. 8 B and not depicted).

Effect of dystroglycan on synaptic localization of laminins

Results presented so far support the idea that laminins $\alpha 4$ or $\alpha 5$ act by concentrating dystroglycan in the postsynaptic membrane, where it can interact with other synaptic components and/or transmit signals to the cell's interior (see Discussion). However, this model would be open to question if dystroglycan were also required for synaptic localization of the laminins, a possibility suggested by studies on myotubes derived from dystroglycan-null embryonic stem cells (Grady et al., 2000). We therefore asked whether dystroglycan is required for the

synaptic localization of laminins $\alpha 4$ or $\alpha 5$ in vivo. In fact, levels and patterns of laminin $\alpha 4$, and laminin $\alpha 5$ immunoreactivity were similar at NMJs *Dag1^{loxP/loxP};Pax3-Cre* mice and littermate controls (Fig. 9, A and B). Likewise, loss of dystroglycan had no detectable effect on the synaptic levels or distribution of integrin $\alpha 7$, integrin $\beta 1$, or Bcam, and patterns of laminin $\alpha 4$, laminin $\alpha 5$, integrin $\alpha 7$, integrin $\beta 1$, and dystroglycan did not differ detectably between *Bcam^{-/-}* mice and controls (Fig. 9, C and D; and unpublished data). The synaptic concentration of laminins at NMJs in *Dag1^{loxP/loxP};Pax3-Cre* muscle suggests that whereas synaptic laminin α chains are required to localize dystroglycan, the opposite is not true.

Discussion

As the NMJ forms, AChRs cluster in the postsynaptic membrane. Early steps in postsynaptic differentiation have been studied in detail and are known to involve interplay of nerve-derived signals and muscle cell-autonomous programs (for review see Kummer et al., 2006). In contrast, little is known about factors that control the subsequent molecular and topological changes that occur as the NMJ matures. Here, we have focused on roles of laminins in the postnatal transformation of the NMJ from a simple plaque to a complex branched array. We analyzed NMJs in a set of five targeted null and conditional mutants, as well as in myotubes cultured aneurally from several of the lines. Collectively with previous studies (see Introduction), our results lead to three major conclusions: (1) laminins containing the $\alpha 4$ and $\alpha 5$ subunits are muscle-derived autocrine promoters of postsynaptic differentiation; (2) these autocrine effects depend in large part on the ability of laminins $\alpha 4$ and $\alpha 5$ in the synaptic cleft to aggregate dystroglycan in the postsynaptic membrane; and (3) the three synaptically concentrated laminin

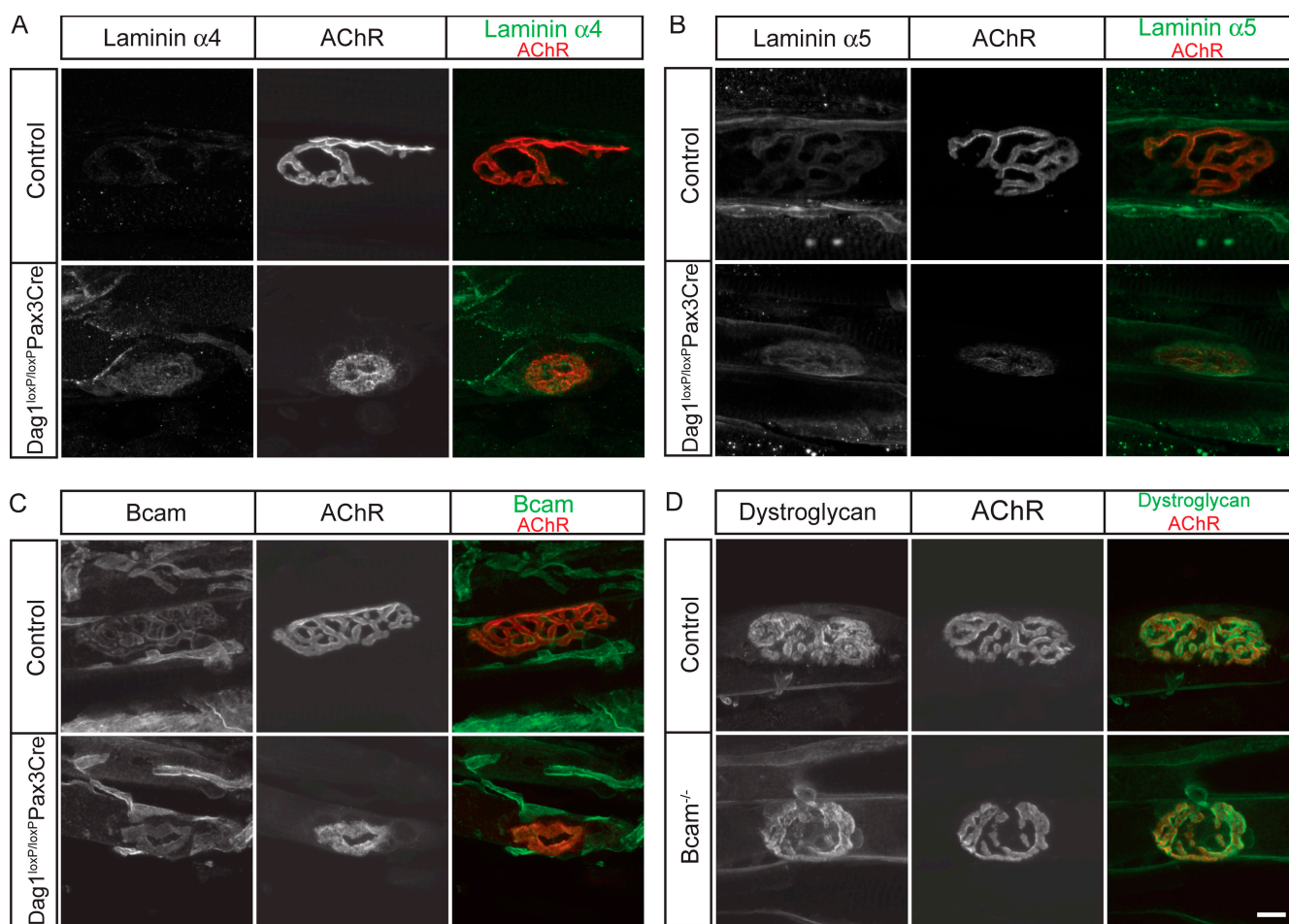


Figure 9. **Roles of dystroglycan in localizing synaptic laminins and Bcam.** (A–C) Laminin α4, laminin α5, and Bcam are similarly concentrated at synaptic sites of control and *Dag1^{loxP/loxP}:Pax3-Cre* muscles. (D) Dystroglycan is similarly concentrated at synaptic sites of control and *Bcam^{-/-}* muscles. Bar, 10 μm.

subunits (α4, α5, and β2) play distinct but interrelated roles in both pre- and postsynaptic differentiation.

Laminins as autocrine synaptic organizing molecules

Pre- and postsynaptic differentiation are interdependent (Sanes and Lichtman, 1999, 2001). Therefore, in considering roles of laminins α4 and α5 in postsynaptic maturation, a main issue is the relationship between their cellular source and target. Three possibilities, which are not mutually exclusive, are: (a) laminins α4 and α5 might be synthesized by nerve or Schwann cells and act transsynaptically on muscle cells; (b) they might be synthesized by muscle and act transsynaptically to affect presynaptic differentiation, with postsynaptic defects being indirect consequences of the presynaptic impairment; and (c) they might both be synthesized by and act directly on the postsynaptic cell.

Several results favor the third of these alternatives, that laminin acts by an autocrine mechanism. First, laminins α4 and α5 are made by myotubes and inserted into basal lamina associated with postsynaptic specializations (Patton, 2000). Second, laminins α4 and α5 are not detectably expressed by motor neurons (Miner et al., 1997). Third, the use of a conditional *Lama5*

mutant allowed us to excise laminin selectively from muscle cells, leaving other potential sources unperturbed. Postsynaptic maturation was delayed in these mice and arrested in mice that also lacked laminin α4. Fourth, myotubes from *Lama5^{MM};Lama4^{-/-}* mice cultured in the absence of neurons displayed defects in AChR aggregation. Thus, although laminins also act on nerve terminals, their effects on postsynaptic maturation are in large part direct.

Several groups have shown previously that application of laminin to cultured myotubes promotes formation of AChR clusters (Vogel et al., 1983; Sugiyama et al., 1997; Montanaro et al., 1998; Smirnov et al., 2002; Marangi et al., 2002). The relationship of this effect to synapse formation in vivo has been unclear, however, because the laminin isoforms used in vitro (laminins 111 and 211) are not found in synaptic basal lamina. Moreover, the effects of soluble laminin in vitro occur in the absence of the receptor tyrosine kinase muscle-specific kinase (MuSK), whereas no AChR clusters form on laminin-laden MuSK^{-/-} myotubes in vivo (e.g., Lin et al., 2001). We have shown that substrate-bound laminin 111 promotes AChR aggregation by a MuSK-dependent mechanism (Kummer et al., 2004) and now show that α4- and α5-containing laminins (421 and 521) are more potent or different from laminin 111.

Dystroglycan as a synaptic laminin receptor

If laminins are autocrine promoters of postsynaptic differentiation, they presumably act through receptors on myotubes. Laminins are large multisubunit proteins, and at least a dozen laminin-binding moieties (proteins and carbohydrates) have been described, many of which are present in muscle (Suzuki, et al., 2005). As one way of narrowing the set of candidates, we took note of the finding that a chimeric laminin, *Mr5G2*, failed to rescue postsynaptic defects on *Lama5*^{-/-} mice (Fig. 2). Because this chimera differs from laminin $\alpha 5$ only in its last three LG domains, we hypothesized that mediators of the effects of laminin on postsynaptic membrane would not only be present in the postsynaptic membrane but also interact with the C-terminal three LG domains of laminin $\alpha 5$. Three receptors that meet these criteria are dystroglycan, Bcam, and $\beta 1$ integrins. Of these, our analysis supports a critical role for dystroglycan.

Dystroglycan was discovered as a laminin-binding protein (Ibraghimov-Beskrovnya et al., 1992; Gee et al., 1993; Barresi and Campbell, 2006), but it was initially implicated in NMJ formation when it was discovered that it also binds agrin, a nerve-derived organizer of postsynaptic differentiation (Bowe et al., 1994; Campanelli et al., 1994; Gee et al., 1994). Later studies showed that agrin does not signal through dystroglycan (Sanes and Lichtman 2001). In parallel, however, evidence accumulated favoring the idea that dystroglycan and other proteins of the dystrophin glycoprotein complex (Grady et al., 1997, 2000) are involved in later aspects of postsynaptic maturation. For example, NMJs in chimeric mice generated from dystroglycan null embryonic stem (ES) cells have a simplified postsynaptic topology (Côté et al., 1999), and cultured myotubes generated directly from such ES cells form abnormal AChR clusters (Grady, et al., 2000; Jacobson et al., 2001). Here, we used conditional dystroglycan mutants to generate mice in which dystroglycan is uniformly deleted from a subset of muscles (*Dag*^{loxP/loxP}; *Pax3-Cre*; unpublished data). We were therefore able to directly compare synaptic morphology in the absence of laminins $\alpha 4$ and $\alpha 5$ and in the absence of dystroglycan. The similar postsynaptic phenotypes in these two mutants supports the idea that dystroglycan mediates the effects of synaptic laminins on postsynaptic differentiation.

Further studies suggest that laminins may act by promoting aggregation of dystroglycan in the postsynaptic membrane, where it can transduce signals or recruit other components. For example, levels of dystroglycan immunoreactivity associated with AChR clusters were markedly reduced in the postsynaptic membrane of *Lama4*^{-/-}; *Lama5*^{M/M} mice and in myotubes cultured aneurally from these mice. Moreover, loss of dystroglycan was not evident at NMJs from *Lama4*^{-/-} or *Lama5*^{M/M} single mutants, in which postsynaptic differentiation was normal or only delayed, respectively. Conversely, laminins $\alpha 4$ and $\alpha 5$ remained concentrated at synaptic sites in dystroglycan-deficient muscle, which indicates that, in vivo, it is laminins that localize dystroglycan rather than vice versa. Together with previous studies showing that soluble laminins can cluster dystroglycan when added to cultured myotubes (Cohen et al., 1997; Montanaro et al., 1998), our results suggest that laminins

are responsible, at least in part, for the synaptic concentration of dystroglycans.

How do laminins $\alpha 4$ and $\alpha 5$ aggregate dystroglycan? The answer is not straightforward, in that dystroglycan also binds laminin $\alpha 2$, which is present throughout the muscle fiber membrane (Fig. 1 E), and laminin $\alpha 1$, which is used as uniform substrate in myotube cultures (Kummer et al., 2004). The simplest possibility is that laminins $\alpha 4$ and $\alpha 5$ bind differently or more tightly to dystroglycan than do laminins $\alpha 1$ and $\alpha 2$. Unfortunately, there is currently no evidence to support this idea, and pure, intact laminin chains are not available to test it. Alternatively, laminins $\alpha 4$ and $\alpha 5$ might recruit linker proteins that aggregate dystroglycan. We have not rigorously tested this possibility but provide evidence that if such linkers do exist, they do not include the laminin- or dystroglycan-binding proteins agrin, integrin $\alpha 7\beta 1$, or Bcam.

How, in turn, might clustered dystroglycan promote postsynaptic maturation? First, one possibility is that it interacts with AChRs to maintain their high density. Indeed, dystroglycan binds to rapsyn, which in turn clusters AChRs, and to MuSK, a component of the primary postsynaptic scaffold (Apel et al., 1995, 1997; Bartoli et al., 2001). Second, dystroglycan is a key component of a dystrophin glycoprotein complex, several intracellular components of which have been implicated in postsynaptic maturation (Deconinck et al., 1997; Grady et al., 1997, 2000, 2003, 2006; Bogdanik et al., 2008). Third, dystroglycan might interact with other laminin receptors such as integrins or Bcam. Our results indicate that Bcam is unlikely to be involved, but we were unable to directly test the role of $\beta 1$ integrins for the postnatal NMJ maturation because deletion of the $\beta 1$ integrin gene from muscle cells leads to embryonic lethality (Schwander et al., 2003, 2004). However, mice lacking integrin $\alpha 7$, the main synaptic laminin-binding integrin, were found to show no morphological abnormalities at the NMJ (Mayer et al., 1997).

Laminins as transsynaptic coordinators

The laminin $\alpha 4$, $\alpha 5$, and $\beta 2$ chains are selectively associated with the basal lamina of the synaptic cleft (Fig. 1 E; Sanes et al., 1990; Patton et al., 1997). Previous studies showed that $\beta 2$ laminins serve as critical organizers of presynaptic maturation (Noakes et al., 1995; Knight et al., 2003; Nishimune et al., 2004) and also affect placement of terminal Schwann cells (Patton et al., 1998). Laminin $\alpha 4$ helps to specify the precise apposition of pre-to-postsynaptic specializations (Patton et al., 2001). Here, we focused on the third synaptically concentrated laminin chain, $\alpha 5$, and discovered new roles of laminins in synaptic maturation. First, analysis of laminin $\alpha 5$ conditional mutants (*Lama5*^{M/M}) showed that $\alpha 5$ is an autocrine promoter of postsynaptic differentiation. Second, analysis of *Lama5*^{-/-}; *Mr5G2* mice showed that laminin $\alpha 5$ acts through a distinct mechanism to ensure complete innervation of the postsynaptic membrane. Third, analysis of *Lama4*^{-/-}; *Lama5*^{M/M} double mutants showed that $\alpha 4$ can substitute for $\alpha 5$, at least in regard to its postsynaptic activity. Together, these results show that synaptic laminins act collaboratively on all three cells of the NMJ to organize and coordinate synaptic maturation.

Materials and methods

Animals

The generation of the following genetically engineered mouse strains has been described previously: *Lama5* null (Miner et al., 1998), *Lama5^{loxP}* conditional mutant (Nguyen et al., 2005), *Lama4* null (Patton et al., 2001), *Lamb2* null (Noakes et al., 1995), *Dag1^{loxP}* conditional mutant (Cohn et al., 2002), *Mr5G2* transgenic mice (Kikkawa et al., 2003; Kikkawa and Miner, 2006), HSA-Cre transgenic mice (Schwander et al., 2003), and Pax3Cre transgenic mice (Li et al., 2000). Generation of *Bcam/Lutheran* null mice was performed by homologous recombination in R1 embryonic stem cells. In brief, the engineered *Bcam* mutation deletes 2 kb of genomic DNA containing exons 11–13 (of 15 total). This segment encodes 142 aa comprising the fifth Ig-like domain, the transmembrane domain, and the first 20 aa of the cytoplasmic tail, so a functional receptor cannot be produced. In addition, splicing from exon 10 to 14 results in an out-of-frame mRNA, and antibodies to either the extracellular or the intracellular domain of Bcam fail to stain *Bcam*^{-/-} mouse tissues (Fig. S5, available at <http://www.jcb.org/cgi/content/full/jcb.200805095/DC1>). All animal studies have been approved by the authors' institutional review boards.

Histological analysis

Primary antibodies used are as follows: anti-agrin (AGR-131; Assay Designs), anti-acetylcholinesterase (gift of T. Rosenberry, Mayo Clinic College of Medicine, Jacksonville, FL), anti- β -dystroglycan (43DAG1/8D5; and 7D11; Novocastra Laboratories, Ltd., and Developmental Studies Hybridoma Bank, respectively), anti-mouse integrin $\alpha 7$ (3C12; Medical and Biological Laboratories Co., Ltd.), anti-mouse $\beta 1$ integrin (MAB1997; Millipore), anti-laminin $\alpha 5$ (Miner et al., 1997), anti-laminin $\alpha 4$, anti-Bcam/Lutheran, anti-laminin $\beta 2$ (rabbit polyclonal antibodies; gifts from T. Sasaki, Oregon Health Sciences University, Portland, OR; and the late R. Timpl, Max-Planck-Institut für Biochemie, Martinsried, Germany; Talts et al., 2000; Sasaki et al., 2002), anti-neurofilament (SM1312; Covance), anti-S100 (polyclonal rabbit; Dako), anti-SV2 (Developmental Studies Hybridoma Bank), Alexa 488-conjugated secondary antibodies, and Alexa 594-conjugated BTX (Alexa 594-BTX; Invitrogen).

Methods for immunohistochemical analysis have been described previously (Kummer et al., 2004; Nishimune et al., 2004). In brief, muscles were fixed in 4% PFA/PBS, sunk in 20% sucrose/PBS, frozen, and cut in a cryostat. Longitudinal sections were cut at 30 μ m and cross sections were cut at 20 μ m. Sections were stained sequentially with primary antibodies and a mixture of Alexa 488-conjugated secondary antibody and Alexa 594-BTX, then mounted in Vectashield (Vector Laboratories). Confocal stacks of 0.5- μ m serial sections were obtained using Fluoview1000 (with Plan Apo 100 $\times$, 1.45 NA or 40 $\times$, 1.3 NA objective lenses; all from Olympus) or C1Si (Plan Apo 60 $\times$, 1.40 NA objective lens; both from Nikon) microscopes. Maximal projection and intensity analysis were performed with Metamorph software (MDS Analytical Technologies). Whole images were level-adjusted, combined to generate color images, and cropped in Photoshop (Adobe). The NMJ was judged as fully occupied when the AChR cluster area did not exceed that of the nerve terminal by more than half the diameter of the axon.

Tissue culture

Primary myotubes were cultured, and morphometric analysis of AChR on cultured myotubes was performed as described previously (Kummer et al., 2004). In brief, limb muscles were dissected from P2–3 mouse, dissociated by trypsin digest and trituration, and cultured on laminin 111 or fibronectin. After 2 d in culture, medium was replaced with reduced serum medium (DME, 5% horse serum, and 10 μ M TTX [Sigma-Aldrich]) and fed fresh medium after 3 d. After 8–10 d in vitro, cells were fixed and stained overnight at 4°C with Alexa 488-labeled BTX, mouse anti- α -dystroglycan (MANDAG2 clone 7D11; Developmental Studies Hybridoma Bank) and rabbit anti-laminin $\alpha 5$ (Miner et al., 1997). Images were collected on a laser scanning confocal microscope (FV1000; Olympus). Plaque and complex AChR clusters were classified as described previously (Kummer et al., 2004) and grouped as follows: plaque-shaped, simple; and perforated/C-shaped/branched, complex. In some cases, recombinant rat agrin (R & D Systems) was added at the final concentration of 2.4 ng/ml to the primary myotubes on the sixth day after switching to the reduced serum medium, and incubated for 11 h in the 37°C CO₂ incubator. Microscopes and objectives were as described in the previous section. To determine dystroglycan levels exclusively at AChR clusters, 1- μ m serial sections were collected and the stacks were maximally projected using Metamorph software. BTX-rich areas were traced, and the traced region was superimposed on the

dystroglycan channel. For each image, the mean extrasynaptic fluorescence was used for background correction.

Evaluation of muscle strength

Muscle strength was evaluated using an inverted screen test (Contet et al., 2001; Cossins et al., 2004). In brief, each mouse was placed in the center of a screen of wire mesh consisting of 1-mm-diameter wires. Then, the screen was smoothly inverted and held ~40 cm above a soft surface. The time until the mouse dropped from the screen was measured. Trials were terminated after 90 s. Each mouse was given five trials.

Online supplemental material

Fig. S1 shows motor impairment of *Lama4*^{-/-};*Lama5*^{MM} mice on the inverted screen test. Fig. S2 shows arrest of postsynaptic maturation in *Lama4*^{-/-};*Lama5*^{MM} mice. Fig. S3 shows that agrin induces AChR clusters on primary myotubes of laminin mutants. Fig. S4 shows small "comet-shaped" AChR clusters in primary muscle cultures. Fig. S5 shows that Bcam staining is absent from NMJs of Bcam null mutant mice. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.200805095/DC1>.

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