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Distinct Target-Derived Signals Organize Formation, Maturation, and Maintenance of Motor Nerve Terminals

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SUMMARY

Target-derived factors organize synaptogenesis by promoting differentiation of nerve terminals at synaptic sites. Several candidate organizing molecules have been identified based on their bioactivities in vitro, but little is known about their roles in vivo. Here, we show that three sets of organizers act sequentially to pattern motor nerve terminals: FGFs, β2 laminins, and collagen α(IV) chains. FGFs of the 7/10/22 subfamily and broadly distributed collagen IV chains (α1/2) promote clustering of synaptic vesicles as nerve terminals form. β2 laminins concentrated at synaptic sites are dispensable for embryonic development of nerve terminals but are required for their postnatal maturation. Synapse-specific collagen IV chains (α3–6) accumulate only after synapses are mature and are required for synaptic maintenance. Thus, multiple target-derived signals permit discrete control of the formation, maturation, and maintenance of presynaptic specializations.

INTRODUCTION

Synapses, which mediate information processing in the nervous system, form at points of contact between axons and their targets. Several features of synapses suggest that their formation is organized by the exchange of developmentally relevant signals between the synaptic partners. For example, pre- and postsynaptic specializations are precisely apposed to each other; pre- and postsynaptic differentiation are temporally as well as spatially coordinated; and the chemical and physiological features of individual synapses (such as the neurotransmitter released by the presynaptic cell and the receptor clustered in the postsynaptic membrane) are matched. Moreover, synaptic specializations often fail to form or mature when axons are experimentally prevented from contacting their targets; in contrast, generation of novel juxtapositions between neurites leads to formation of synapses at points of ectopic contact (Sanes and Lichtman, 1999; Fox and Umemori, 2006).

Based on these observations, many groups have sought synaptic organizing molecules, generally using cultured neurons to assay bioactivity. Over the past decade, several have been identified. Focusing on molecules that promote presynaptic differentiation, these include the membrane-associated adhesion and signaling molecules neuroligin, SynCAM, and Eph kinases; the extracellular matrix components thrombospondin and laminin β2; members of the Wnt and fibroblast growth factor (FGF) families of secreted differentiation factors; and cholesterol (Noakes et al., 1995; Biederer et al., 2002; Dean et al., 2003; Umemori et al., 2004; Christopherson et al., 2005; Goritz et al., 2005; Ahmad-Annuar et al., 2006; reviewed in Ziv and Garner, 2004; Craig et al., 2006; Fox and Umemori, 2006).

This embarrassment of riches leads to a new question: Do organizers with similar effects in vitro play distinct roles in vivo? One possibility is that different synapses use

different organizers in vivo even though each is capable of affecting a variety of neuronal types in vitro. Second, different organizers might promote distinct aspects of differentiation such as recruitment of ion channels or clustering of synaptic vesicles. Third, multiple organizers could act combinatorially, allowing fine control of synaptogenesis. Fourth, organizers may act sequentially, with different molecules regulating the initial development, subsequent maturation, and dynamic maintenance of the synapse. Fifth, some factors capable of promoting presynaptic differentiation in culture may have different functions in vivo, such as regulation of synaptic efficacy. Clearly, many other possibilities exist.

Here, we use the mouse neuromuscular junction (NMJ) to address this issue. This synapse is large and experimentally accessible, and its development has been analyzed in detail (Sanes and Lichtman, 1999; Kummer et al., 2006). Using targeted mouse mutants, we show that members of three gene families (FGF, laminin, and collagen IV) all play roles at the NMJ. Although their effects on cultured motoneurons are superficially similar, they have distinct and sequential effects in vivo: FGFs and collagen α1/2(IV) chains direct the initial differentiation of nerve terminals, β2 laminins promote their maturation, and collagen α3–6(IV) chains are required to maintain them. Thus, one rationale for the existence of multiple organizers is to permit separate control of distinct phases in the life of a synapse. Finally, we provide evidence that developmental regulation of the organizers' expression accounts at least in part for the timing of their effects.

RESULTS

FGF-Dependent Presynaptic Differentiation in Nerve-Muscle Cocultures

A critical step in presynaptic differentiation is the clustering of synaptic vesicles within varicosities. We found recently that three closely related members of the FGF family, FGF7, -10, and -22, promote vesicle clustering in several neuronal types in vitro and in pontine and in vestibular axons in vivo (Umemori et al., 2004). To begin the present study, we asked whether FGFs also mediate formation of synaptic varicosities at NMJs formed in vitro between embryonic spinal motoneurons and cells of the C2 myogenic line. RT-PCR showed that C2 cells cultured alone expressed FGF7, -10, and -22 (Figure 1A). FGF7, -10, and -22 signal through an alternatively spliced form of FGF receptor 2 (FGFR2) called FGFR2b; they activate the FGFR2c isoform poorly if at all (Zhang et al., 2006). Motoneurons cultured alone expressed both FGFR2b and FGFR2c, whereas muscle cells expressed FGFR2c but not FGFR2b (Figures 1A–1C). When the two cell types were cocultured, motoneurons extended neurites that contacted C2 myotubes and formed synaptic vesicle-rich varicosities at these sites (Figure 1D). To ask whether FGF7/10/22 are presynaptic organizers in this system, we generated soluble proteins in which the extracellular domains of FGFR2b or FGFR2c were fused to alkaline

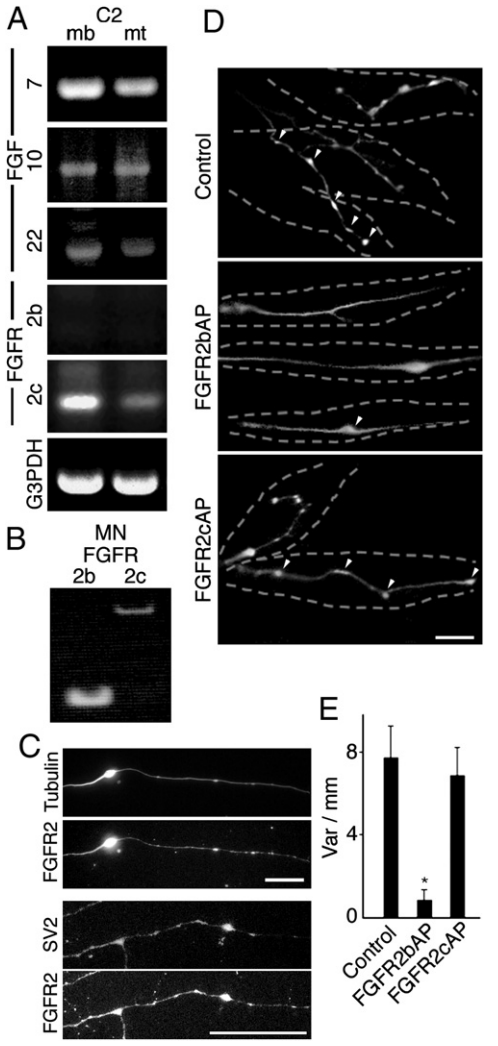

Figure 1. FGF-Dependent Presynaptic Differentiation in Cultured Motoneurons

(A) RT-PCR analysis shows that FGF7, FGF10, FGF22, and FGFR2c are all expressed by both C2 myoblasts (mb) and myotubes (mt). FGFR2b is not expressed by either myoblasts or myotubes. Glyceraldehyde 3-phosphate dehydrogenase (G3PDH) was used as a control.

(B) RT-PCR analysis shows that FGFR2b and -2c are both expressed by isolated embryonic motoneurons.

(C) FGFR2 is present on motoneuron neurites. Cultured motoneurons were stained for FGFR2 and either acetylated tubulin or the synaptic vesicle protein SV2.

(D) Motoneurons and myotubes were cocultured for 3 days with FGFR2bAP, FGFR2cAP, or control protein (AP) then stained for synapsin. FGFR2bAP, which sequesters FGF7, -10, and -22, but not FGFR2cAP, prevents formation of vesicle-rich varicosities at sites of neurite-myotube contact. Dotted lines indicate the edges of myotubes. Arrowheads indicate all varicosities on a single neurite in the field of view.

(E) Quantitation of results from cocultures such as those in (D). Bars show mean ± SEM for 100 neurites per condition. *: differs from control and from FGFR2cAP at $p < 0.01$ by Tukey test. Var indicates vesicle-rich varicosities.

Bar is 75 μm in (C) and (D).

phosphatase (AP). FGFR2bAP binds to and thereby neutralizes FGF7/10/22, while FGFR2cAP serves as a control (Ornitz et al., 1992; Umemori et al., 2004). Neither protein had any detectable effect on neurite elongation (control: 470 ± 21 μm; FGFR2bAP: 449 ± 19 μm; FGFR2cAP: 453 ± 52 μm; mean ± SEM, n = 21) or myotube differentiation (data not shown). However, FGFR2bAP reduced the incidence of varicosities at neurite-myotube contacts by >80% (Figures 1D and 1E). These results suggest that FGFs of the 7/10/22 subfamily are target derived mediators of presynaptic differentiation at the NMJ.

FGF-Dependent Presynaptic Differentiation at Embryonic NMJs

In vivo as in vitro, developing motoneurons express FGFR2, and muscles express all three members of the FGF7/10/22 subfamily (Figures 2H and 2I). Because all of these FGFs might act as target-derived organizers, we tested their role by using an isoform-specific targeted mutant that selectively inactivates FGFR2b without detectably affecting expression of FGFR2c (Eswarakumar et al., 2002; Figure 2A). Synaptic vesicles were stained with antibodies to synaptophysin, synaptotagmin, or synapsin; axons were stained with antibodies to neurofilaments; and acetylcholine receptor (AChR)-rich postsynaptic sites on myotubes were stained with α-bungarotoxin (BTX).

In wild-type mice, synaptic vesicles are initially present throughout the motor axon but become progressively concentrated at synaptic sites as terminals form in apposition to AChR clusters (Lupa et al., 1990). In FGFR2b$^{-/-}$ mice, axons entered muscles normally and arborized at AChR-rich sites. Moreover, numerous vesicles were present in motor axons of these mutants (Figures 2C–2E and S1). By E16, however, vesicles were less restricted to synaptic sites in FGFR2b$^{-/-}$ muscles than in controls (Figures 2B and S1). The increased density of vesicles in mutants was striking in distal nerve branches near synaptic sites but minimal in proximal regions and in main nerve trunks (Figure 2C). Western blotting demonstrated similar levels of synapsin in diaphragms and phrenic nerves of E18 mutants and littermate controls (data not shown). These results suggest that FGF signaling through FGFR2b is dispensable for vesicle formation and transport but is required for their local accumulation in nerve terminals.

FGFR2b$^{-/-}$ mutants die at birth due to lung defects (De Moerlooze et al., 2000), so we were unable to assess the effect of FGF7/10/22 signaling postnatally. To circumvent this limitation, we used a conditional FGFR allele (FGFR2^{flox}) in which all isoforms of FGFR2 can be deleted from specific cell types by Cre-mediated excision (Yu et al., 2003). Here, we used mice that expressed Cre recombinase under the control of regulatory elements from the HB9 or Islet 1 (Isl) genes, which restrict expression of Cre to a small subset of cell types including motoneurons (Figure 2F). We verified motoneuronal expression of Cre in these mice by mating them to mice that express YFP in neurons following Cre-mediated excision of the stop cassette (Buffelli et al., 2003; data not shown).

FGFR2$^{flox/flox}$; HB9-Cre and FGFR2$^{flox/flox}$; Isl-Cre mice were born in expected numbers. They were smaller than littermate controls and exhibited a mild tremor but otherwise were outwardly normal. At embryonic stages, the neuromuscular phenotypes of FGFR2$^{flox/flox}$; HB9-Cre and FGFR2$^{flox/flox}$; Isl-Cre mice were indistinguishable from those described above for FGFR2b$^{-/-}$ mice: NMJs formed on schedule, but vesicles were less concentrated at synaptic sites in mutants than in controls (Figures 2G and S2). A similar defect was observed in mutant neonates and during the first postnatal week (extrasynaptic/synaptic ratio, calculated as in Figure 2B, 0.44 ± 0.02, n = 45 for control and 0.60 ± 0.03, n = 60 for mutants at P0; mean ± SEM; $p < 0.0001$). Remarkably, however, the abnormality was transient: it was less striking by P7 than at birth and barely detectable during the third postnatal week (extrasynaptic/synaptic ratio, 0.31 ± 0.02 for control and 0.36 ± 0.07 for mutants at P17; Figures 2G and S2). Thus, FGF signaling is required for clustering of synaptic vesicles in embryos, but other mechanisms can eventually promote clustering independent of FGFR2.

What accounts for the transient nature of the neuromuscular phenotype in FGFR2 mutants? One possibility is that expression of FGF7/10/22 by muscles is transient, with other organizers taking their place later. Indeed, levels of FGF7, -10, and -22 mRNAs declined during the first postnatal week and were barely detectable by P21 (Figure 2H). In addition, anti-FGFR2 immunoreactivity was present at relatively high levels at synaptic sites in fetal muscle, but levels declined postnatally (Figure 2I). Loss of staining following denervation showed that immunoreactivity was associated with nerve terminals (Figure S3A); its absence in FGFR2$^{flox/flox}$; HB9-Cre mice showed that it was specific (Figure S3B). Thus, developmental regulation of FGF ligands in muscle and their receptor in motor nerve terminals provides a partial explanation for the transient neuromuscular phenotype of FGFR2 mutants.

Laminin β2-Dependent Presynaptic Maturation at Postnatal NMJs

The transient requirement for FGF signaling implies that additional factors act subsequently to FGFs to promote clustering of vesicles at synaptic sites and/or compensate for the loss of the FGF signaling. β2 laminins (α/β/γ heterotrimers containing the β2 subunit) are reasonable candidates for such additional factors. Laminins composed of α2, α4, or α5 plus β2 and γ1 subunits are synthesized by muscle and concentrated in the basal lamina that forms the synaptic cleft material at the NMJ; extrasynaptic portions of the basal lamina are rich in β1 but poor in β2 laminins (Patton et al., 1997). β2 laminins promote presynaptic differentiation in vitro (Son et al., 1999; Nishimune et al., 2004), and mice lacking laminin β2 exhibit defects in presynaptic differentiation that lead to lethality during the third postnatal week (Noakes et al., 1995; Knight et al., 2003). Neuromuscular failure in LAMB2$^{-/-}$ null mice

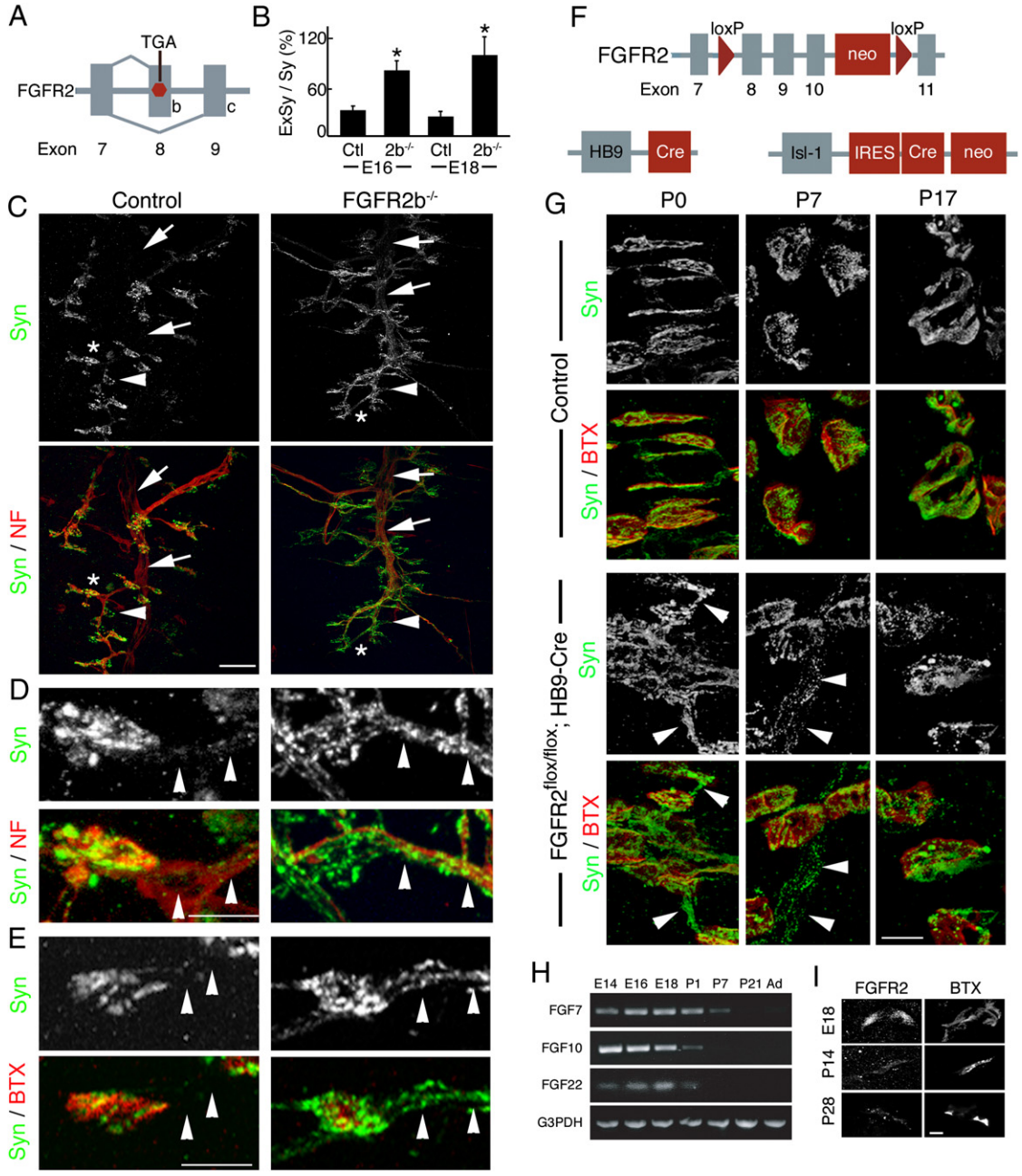

Figure 2. FGF-Dependent Presynaptic Differentiation at the Embryonic NMJ

(A) Schematic representation of the FGFR2b-specific mutant allele. Splicing of exon 7 to exons 8 and 9 generates 2b and 2c variants, respectively. To specifically inactivate FGFR2b without affecting FGFR2c expression, a stop codon (TGA) was generated in exon 8.

(B) Quantitation of synaptic vesicles failing to correctly aggregate in nerve terminals of E16 and E18 control and FGFR2b$^{-/-}$ mutant NMJs. Ratio of levels of extrasynaptic to synaptic (ExSy/Sy) synaptophysin staining is shown. Bars show mean ± SEM for at least 8 NMJs per condition. *: differs from age-matched control at $p < 0.01$ by Student's t test.

(C–E) Inactivation of FGFR2b diminishes clustering of synaptic vesicles in motor nerve terminals. Whole triangularis sterni muscles (C–D) and diaphragm (E) from E18 FGFR2b$^{-/-}$ and control embryos were stained with antibodies to synaptophysin (Syn) plus antibodies to neurofilament (NF) to show axons (C–D) or with BTX to show postsynaptic sites (E). Terminals starred in (C) enlarged in (D). Vesicle restriction to control terminals is striking by E18, while vesicles are present in distal (arrowheads) but not proximal (arrow) preterminal portions of mutant axons.

(F) Schematic representation of FGFR2$^{flox/flox}$ conditional allele and two transgenic lines, HB9-Cre and Isl-Cre, which express Cre selectively in motoneurons. Cre excises exons 8–10 to inactivate FGFR2.

(G) FGFR2 mutant neuromuscular phenotype decreases postnatally. Diaphragms from control and FGFR2$^{flox/flox}$; HB9-Cre mice were stained for synaptophysin and BTX at the ages indicated. Arrowheads indicate preterminal axons.

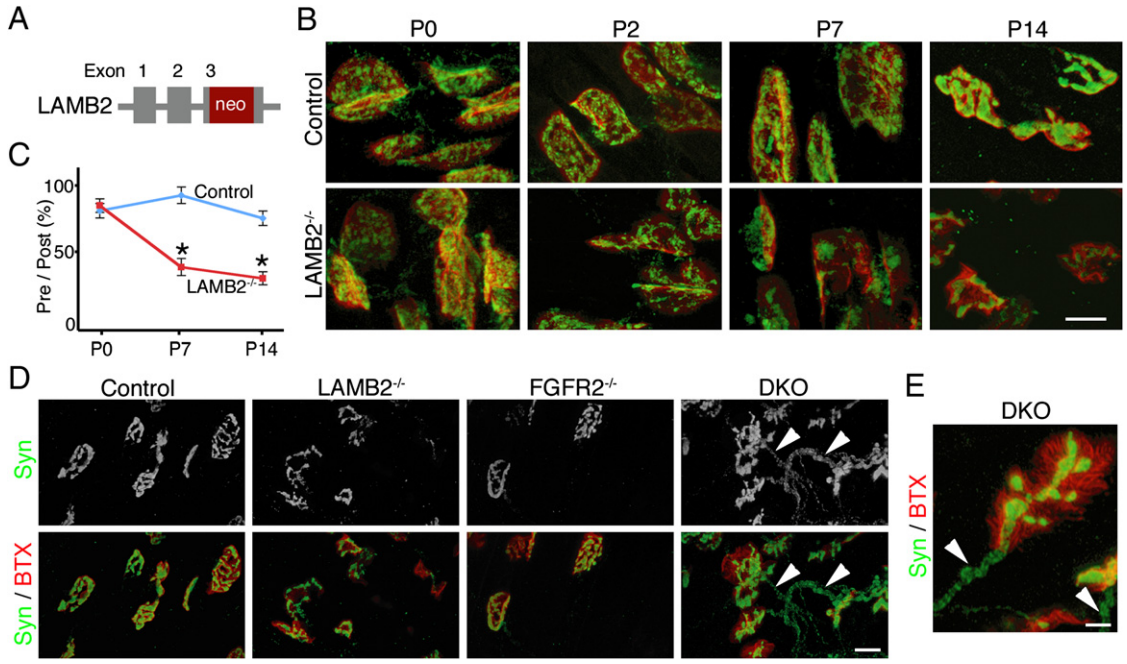

Figure 3. Laminin β2-Dependent Presynaptic Maturation at the Postnatal NMJ

(A) Schematic representation of the LAMB2 mutation. A neomycin cassette replaces the second coding exon (exon 3), generating a null allele.

(B) Laminin β2 neuromuscular phenotype appears postnatally. Diaphragms from LAMB2$^{-/-}$ and control mice were stained for synaptophysin (green) and BTX (red) at ages indicated. Levels of synaptophysin are similar at neonatal mutant and control NMJs, but levels in mutants fail to increase during development as they do in controls.

(C) Quantitation of the percentage of postsynaptic membrane covered by presynaptic terminals in P0, P7, and P14 control and LAMB2$^{-/-}$ NMJs. Bars show mean ± SEM for at least 10 NMJs per condition. *: differs from age-matched control at $p < 0.01$ by Student's t test.

(D) Persistence of vesicles in preterminal axons (arrowheads) of FGFR2$^{flox/flox}$; HB9-Cre; and LAMB2$^{-/-}$ double mutants (DKO; P14, diaphragms, stained as in B). In contrast to the DKO, few vesicles are seen in preterminal portions of axons in controls or in either single mutant at this age.

(E) High magnification of DKO demonstrating persistence of vesicles in preterminal axons (arrowheads).

Bars are 3.3 μm in (B), 4 μm in (D), and 1 μm in (E).

results from lack of muscle-derived laminin β2, as defects can be rescued by selective transgenic expression of laminin β2 in muscle (Miner et al., 2006).

To assess the possibility that FGFs and β2 laminins regulate presynaptic differentiation sequentially, we examined neonatal laminin β2 null mutants (LAMB2$^{-/-}$; Figure 3A). The size of nerve terminals in diaphragm and the apparent density of vesicles in terminals did not differ detectably between LAMB2$^{-/-}$ mice and littermate controls during the first few postnatal days (Figure 3B). By P7, however, mutant NMJs were smaller than those in controls, the density of vesicles was reduced, and the fraction of the postsynaptic membrane covered by nerve terminals was significantly decreased (Figures 3B and 3C). Similarly, the density of active zones in control and mutant nerve terminals was similar at birth but was significantly greater in controls than in mutants by the end of the first postnatal week (data not shown; Nishimune et al., 2004). Thus, laminin β2 is required for postnatal but not prenatal presynaptic differentiation.

To examine whether the presence of laminin β2 accounted for the recovery of the synaptic defects observed in FGFR2 mutants, we used mutants lacking both presynaptic organizers. If laminin β2 compensates for the absence of FGFR2, one would expect a more severe neuromuscular phenotype in double mutants than in either single mutant. Vesicles were highly clustered at synaptic sites in both FGFR2$^{flox/flox}$; HB9-Cre; and LAMB2$^{-/-}$ mice at P14 but were abundant in preterminal portions of double mutants (Figures 3D and 3E; extrasynaptic/synaptic ratio, 0.31 ± 0.02 for control, 0.70 ± 0.05 in double mutant at P14; $p < 0.00001$). Thus, FGF and laminin β2 signals may functionally compensate for each other at the NMJ.

(H) RT-PCR analysis of leg muscles shows postnatal decline in FGF7, -10, and -22 mRNA levels. Glyceraldehyde 3-phosphate dehydrogenase (G3PDH) was used as a control.

(I) Immunofluorescence shows FGFR2 associated with motor nerve terminals at BTX-rich synaptic sites. Levels are high at E18 then decline postnatally (P14 and P28).

Bars are 10 μm in (C–E), 12 μm for (G), and 5 μm in (I).

Identification of Additional Organizing Factors

Presynaptic differentiation proceeds to a considerable extent at the NMJ in the absence of both FGFR2 and laminin β2, suggesting that additional organizers exist. To identify such factors, we used the electric organ of *Torpedo californica*. This tissue is rich in myocyte-related electrocytes, one side of which is almost completely covered by motor nerve terminals. Due to the large size of these synapses and the paucity of contractile proteins, electric organs serve as an exceptionally rich source of NMJ components. Knowing that some synaptic organizing molecules persist in the adult electrocyte (Nitkin et al., 1987; Sunderland et al., 2000), we applied to *Torpedo* electric-organ methods previously used to isolate FGF22 from mouse brain (Umemori et al., 2004).

Briefly, extracts of electric organ were applied to purified motoneurons in low-density culture, and fractions were sought that led to clustering of synaptic vesicles in neurites, as assessed by staining for synapsin. Proteins that were solubilized at high ionic strength from an extracellular matrix-rich fraction not only promoted vesicle clustering but also increased both neurite length and neurite branching (Figure 4A). We fractionated the extract chromatographically, using vesicle clustering as a bioassay. Weak anion-exchange chromatography (DEAE) revealed a peak of vesicle-clustering activity eluting at 100 mM NaCl that had no detectable effect on neurite length or branching (data not shown). Proteins in this active fraction were further fractionated by successive steps of strong anion-exchange chromatography (HiTrap Q), gel filtration (Superdex 200), and cation-exchange chromatography (MONO-S; Figure 4B; data not shown). The most active fractions following the MONO-S column were analyzed by SDS-PAGE. The only visible proteins appeared as a triplet ranging from 22 to 28 kDa (Figure 4C); neighboring fractions with diminished activity contained little or none of this triplet.

The three bands were excised separately from gels and analyzed by tandem mass spectrometry. Peptides in all three bands contained sequences with glycine at every third residue (GXY repeats), suggesting that the active material was collagenous (Table S1). The same collagen-like sequences were present in all three bands, suggesting that all contained proteins derived from the same gene or genes. Because similar GXY repeats are present in >60 mammalian collagen-like chains and the *Torpedo* genome remains unsequenced, we could not match these *Torpedo* sequences to particular genes. However, the 28 kDa band contained three noncollagenous sequences that were highly similar (>90% identity; see Table S1) to sequences in the noncollagenous carboxy-terminal (NC1) domains of chick and mammalian collagen α(IV) chains (Miner and Sanes, 1994; Oohashi et al., 1995). To verify the identity of the purified material, we used an antibody that recognizes NC1 domains of multiple collagen α(IV) chains. The antibody reacted with all three bands in the active fraction, showing that all contained NC1-derived epitopes (Figure 4D). These results suggest that fragments of *Torpedo* collagen IV are capable of clustering vesicles in motor axons.

Presynaptic Organizing Activity of Collagen α2, α3, and α6(IV) NC1 Domains

The vesicle-clustering proteins we isolated contained epitopes derived from both NC1 and collagenous domains. Recent studies have demonstrated biological activities in NC1 domains of several collagens (reviewed in Ortega and Werb, 2002). We therefore asked whether synaptic organizing activity resided in the NC1 domain of a collagen IV chain. The fraction analyzed (which had been purified on DEAE, Q, and Superdex but not MONO-S columns) contained several larger bands not seen in the most pure fractions. These bands also reacted with the anti-NC1 antibody (Figure 4E), and sequencing showed that they contained some of the same collagenous and noncollagenous peptides present in the more pure fractions as well as additional collagenous sequences (Table S2). Treatment of this fraction with collagenase, which digests GXY sequences, led to a loss of all pre-existing bands and the generation of a predominant 20 kDa NC1-reactive band plus a weaker 60 kDa band. These bands likely represent a single NC1 domain and an SDS-resistant multimer, respectively (Borza et al., 2001; Figure 4E). Collagenase-treated fractions retained the ability to induce synapsin aggregation in chick motoneurons (Figure 4F). These results suggest that the active polypeptides consist of NC1 domains linked to collagenous sequences of variable lengths and that their ability to cluster synaptic vesicles resides in the NC1 domains.

Mammalian genomes contain six genes that encode collagen α(IV) chains: COL4A1–COL4A6 (Hudson et al., 2003). We assessed the effects of all six recombinant human collagen IV NC1 domains on motoneurons to determine whether NC1 domains were sufficient to cluster vesicles and which were active. Recombinant α2, α3, and α6 NC1 domains clustered vesicles, whereas α1, α4, and α5 NC1 domains were inactive in this assay (Figure 4G). In light of the finding that collagen IV stabilizes AChR clusters induced by soluble factors in a myogenic cell line (Smirnov et al., 2005), we also asked whether NC1 domains could induce or stabilize AChR clusters and found that they could not (Figure S4).

Collagen α1/2(IV)-Dependent Presynaptic Differentiation

Collagen α1/2(IV) are present throughout the basal lamina in adult muscle fibers, whereas the α3–5 chains are present only at synaptic sites (Miner and Sanes, 1994). We used a panel of chain-specific antibodies to ask when each chain appeared. The α1/2 chains are present throughout the embryonic myotube basal lamina and remain present in both the synaptic and extrasynaptic basal lamina throughout life. In contrast, the α3–6 chains are undetectable in embryonic basal lamina and do not appear at synaptic sites until the third postnatal week (Figure 5A; data not shown).

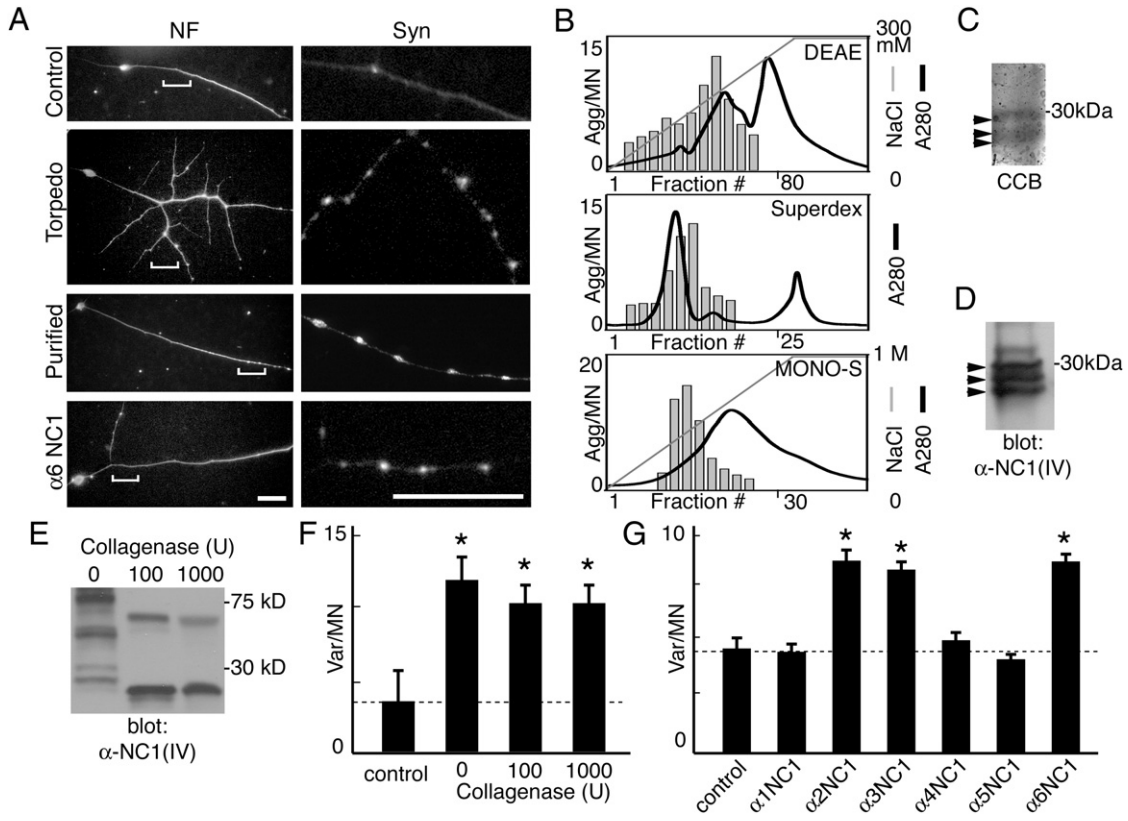

Figure 4. Collagen IV Is a Presynaptic Organizing Molecule

(A) Cultured motoneurons (MNs) stained with anti-neurofilament (NF) to visualize neurites and synapsin (Syn) to assess the distribution of synaptic vesicles. Prior to staining, cultures were incubated 48 hr in control medium in the presence of *Torpedo* electric organ extract, purified active material from *Torpedo* (MONO-S column; see B), or human recombinant α6 NC1 domain of collagen IV (~5 μg/ml). The crude electric organ extract promoted neurite elongation and branching as well as synaptic vesicle aggregation. The MONO-S-purified protein and α6 NC1 domain also promoted synaptic vesicle clustering but had little effect on neurite length or branching. Brackets indicate areas enlarged to demonstrate synapsin staining. Bars are 25 μm.

(B) Purification of vesicle-clustering material through sequential DEAE, Q (not shown), Superdex, and MONO-S columns. Activity (bars) was assayed as in (A) and expressed as the number of vesicle aggregates per neuron. Protein concentration was monitored by absorbance at 280 nm (black lines). Gray line shows NaCl concentration of eluate.

(C) The most active fractions from the MONO-S column were pooled, separated by SDS-PAGE, and stained with Coomassie Colloidal Blue. Bands indicated with arrowheads were excised for sequencing.

(D) The most active fractions from the Superdex separation were subjected to immunoblotting with an antibody that recognizes the NC1 domain of multiple collagen α(IV) chains. The three immunoreactive bands at ~22–28 kDa (arrowheads) correspond to the major protein components in (C).

(E) Treatment of an active fraction from Superdex separation with 100 or 1000 units of collagenase reduced most of the NC1-positive bands to ~20 kDa or ~60 kDa.

(F) Collagenase-treated material retained its vesicle-clustering activity when assayed as in (A) and (B). Control solution contained 1000 U collagenase. Var indicates vesicle-rich varicosities. The dashed line indicates levels of controls. Bars show mean ± SEM for three independent experiments. *: differs from controls at $p < 0.05$ by ANOVA.

(G) Motoneurons were treated for 48 hr with 5 μg/ml recombinant human collagen NC1 domains as in (A). The α2, α3, and α6 NC1 domains induced vesicle clustering. Var indicates vesicle-rich varicosities. The dashed line indicates levels of controls. Bars show mean ± SEM for five experiments. *: differs from controls at $p < 0.01$ by ANOVA.

To initiate analysis of collagen IV in vivo, we first focused on the early-appearing α1/2 chains. Collagen α(IV) chains are secreted in the form of trimers. Although in principle the six collagen α(IV) chains could assemble into >50 homo- and heterotrimers, biochemical analysis indicates that only three combinations exist: $(\alpha1)_2(\alpha2)$, $(\alpha3)(\alpha4)(\alpha5)$, and $(\alpha5)_2(\alpha6)$ (Figure 5B; Boutaud et al., 2000; Borza et al., 2001). In the absence of any single chain, the inability to assemble the corresponding trimer leads to the absence of the other chains from the basal lamina (Hudson et al., 2003). Thus, neither α1 nor α2 chains are present in basal laminae of COL4A1 mutants. Although null COL4A1 mutant embryos die before any synapses form (Poschl et al., 2004), a viable COL4A1 mutant was recently identified in which a small deletion decreases trimer assembly by a dominant-negative mechanism

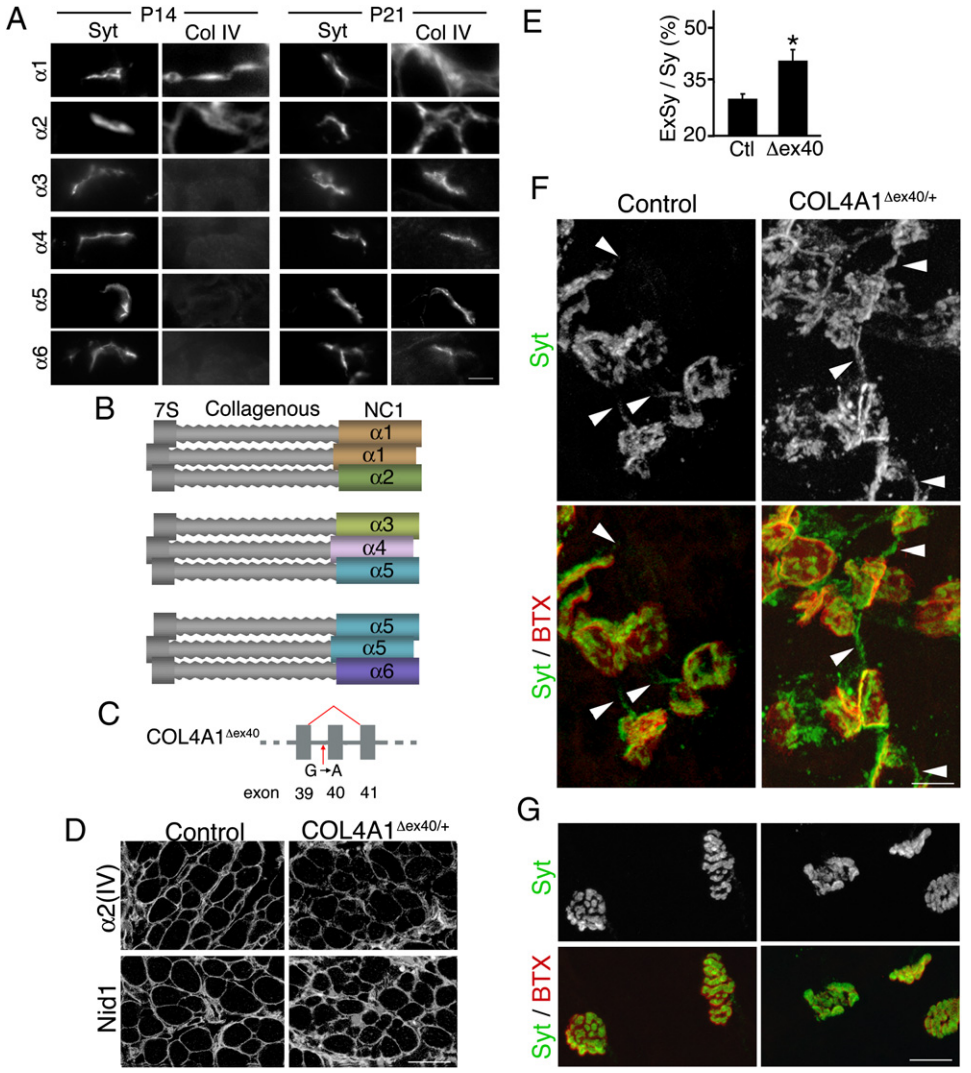

Figure 5. Collagen IV-Dependent Formation of the NMJ

(A) Collagen α1(IV) and α2(IV) chains are present throughout the myotube's basal lamina from birth, whereas collagen α3–6(IV) chains appear specifically at the NMJ during the third postnatal week.

(B) The six collagen α(IV) chains assemble into only three trimers. Gray boxes indicate the N-terminal 7S domain; gray rods indicate the collagenous domain; and other colors indicate NC1 domains.

(C) Schematic representation of COL4A1$^{\Delta ex40}$ mutant. A single nucleotide exchange in the splice acceptor site of exon 40 (G to A) causes a deletion of exon 40, leading to production of a protein that blocks trimer assembly and secretion.

(D) Reduction of collagen α2 (IV) in P3 COL4A1$^{\Delta ex40}$ mutant muscle. Nidogen 1 (entactin) levels appear unchanged.

(E) Quantification of synaptic vesicles failing to correctly aggregate in nerve terminals of P3 control and COL4A1$^{\Delta ex40}$ mutant NMJs. Ratio of levels of extrasynaptic to synaptic synaptotagmin 2 staining (ExSy/Sy) is shown. Bars show mean ± SEM for at least 41 NMJs per condition. *: differs from age-matched control at $p < 0.01$ by Student's t test.

(F) NMJs from COL4A1$^{\Delta ex40}$ mutant and littermate controls at P3 stained with antibodies to synaptotagmin 2 (Syt) and BTX. Arrowheads indicate preterminal portions of axons. Synaptic vesicles are less restricted to synaptic portions of nerve terminals in mutants.

(G) NMJs from COL4A1$^{\Delta ex40}$ mutant and littermate controls at P21 stained with antibodies to synaptotagmin 2 (Syt) and BTX. No defects are detectable in mutants after the first 3 postnatal weeks.

Bar is 5 μm in (A), (D), (F), and (G).

(COL4A1$^{\Delta ex40}$; Gould et al., 2005, 2006; Figure 5C). We used COL4A1$^{\Delta ex40/+}$ mice to assess the role of collagen α1/2(IV) in nerve terminal formation.

Muscles formed normally in COL4A1$^{\Delta ex40/+}$ mutants. Levels of collagen α2(IV) were decreased, but the basal lamina was intact, as shown by normal staining with antibodies to nidogen 1 (Figure 5D). In P3 heterozygotes, the density of synaptic vesicles was increased in preterminal portions of motor axons (Figures 5E and 5F), as described above for FGFR mutants. In addition, sprouts extended

from nerve terminals more frequently in mutants than in controls (data not shown). However, by the third postnatal week synaptic phenotypes were no longer observed (Figure 5G). This recovery suggests that either enough collagen α1/2(IV) eventually accumulates postnatally in the mutant basal lamina to allow for proper nerve terminal maturation or that other factors such as laminin β2 or the collagen α3/6(IV) chains are capable of compensating.

Collagen α3–6(IV)-Dependent Maintenance of Nerve Terminals

The selective association of the collagen α3–6(IV) chains with synaptic sites (Figure 5A) taken together with the known rules of trimer assembly (Figure 5B) implies that (α3)(α4)(α5) and $(\alpha5)_2(\alpha6)$ trimers are present at the NMJ. To test the role of these chains in NMJ formation in vivo, we used a set of three targeted mutants: COL4A3$^{-/-}$, COL4A5$^{-/Y}$, and COL4A6$^{-/Y}$ (Figure 6A; COL4A5 and COL4A6 are on the X chromosome). We predicted that α3 and α4 chains would be absent from the NMJs in COL4A3$^{-/-}$ mutants, that α3–6 chains would be absent in COL4A5$^{-/Y}$ mutants, and that only the α6 chain would be absent in COL4A6$^{-/Y}$ mutants (Figure 6B). We confirmed these predictions by immunostaining of COL4A3$^{-/-}$, COL4A5$^{-/Y}$, and COL4A6$^{-/Y}$ muscle (Figure S5 and data not shown). Thus COL4A3$^{-/-}$ and COL4A6$^{-/Y}$ mutants each lack one of the two synapse-specific collagen α(IV) chains with vesicle-clustering activity (α3 and α6), whereas COL4A5$^{-/Y}$ mutants lack both of these chains.

COL4A6$^{-/Y}$ mice are healthy, fertile, and display no known defects (Y. Ninomiya, personal communication). COL4A3$^{-/-}$ and COL4A5$^{-/Y}$ appear healthy at birth, but die at 6-32 weeks of age due to renal defects (Miner and Sanes, 1996; Cosgrove et al., 1996; Rheault et al., 2004). NMJs in COL4A3$^{-/-}$ or COL4A6$^{-/Y}$ muscles, which lack either the α3 or α6 chain, respectively, did not differ detectably from those in littermate controls (Figure S6). Likewise, no nerve-terminal defects were observed in COL4A5$^{-/Y}$ muscle during the first three postnatal weeks, as expected from the late appearance of the collagen α3–6(IV) chains (Figures 6C, S7A, and S7B). In 1-month-old COL4A5$^{-/Y}$ diaphragms, however, axons had retracted from portions of the AChR-rich postsynaptic membrane (Figure S7C). By 2 months of age, about half of the NMJs were aberrant (Figures 6D–6G). First, whereas NMJs in control muscle are composed of branches arranged in a pretzel-like configuration, pre- and postsynaptic specializations are often fragmented in COL4A5$^{-/Y}$ NMJs. Second, whereas nerve terminals completely covered AChR-rich postsynaptic sites in control NMJs, some AChR-rich fragments were unapposed by nerve terminals in ~25% of COL4A5$^{-/Y}$ NMJs (21/80; arrowheads in Figure 6D). Third, neurofilaments formed ring-like structures in nerve terminals at some COL4A5$^{-/Y}$ NMJs (arrows in Figures 6F and 6G). Similar structures are sometimes seen in immature control NMJs and resemble microtubule-rich loops recently described in maturing *Drosophila* NMJs (Roos et al., 2000). Finally, whereas motor axons are cylindrical in controls, the preterminal axon segments were often distended at COL4A5$^{-/Y}$ synaptic sites (arrowheads in Figure 6F).

Several observations suggested that these defects were due to the loss of both collagen α3(IV) and α6(IV) from synaptic sites in the mutant. First, as noted above, we detected no defects at NMJs in diaphragms of COL4A3$^{-/-}$ or COL4A6$^{-/Y}$ mice, which lack α3 or α6, respectively. Second, loss of the α3–6 chains did not lead to a general disruption of the basal lamina, as levels of collagen α1(IV), collagen α2(IV), and laminin β2 appeared normal at NMJs in COL4A5$^{-/Y}$ muscles (Figure S5 and data not shown). Third, synaptic defects were not secondary to the renal defects since lethal renal failure occurs in both COL4A3$^{-/-}$ and COL4A5$^{-/Y}$ mutants (Miner and Sanes, 1996; Rheault et al., 2004), but synaptic defects were detected only in the latter. Finally, synaptic defects were not secondary to myopathy since central nuclei (which mark fibers that have degenerated and regenerated) were rarely observed in COL4A5$^{-/Y}$ muscles (Figure S8). Together, these results indicate that the two synapse-specific collagen IV chains capable of clustering vesicles in cultured motoneurons also affect NMJ architecture in vivo and that the α3 and α6 chains have overlapping or redundant functions at the synapse.

DISCUSSION

Over the past decade, several molecules have been identified that, when applied to cultured neurons, are capable of promoting at least some steps in the transformation of neurites into functional nerve terminals. These results led us to ask what roles molecules with similar in vitro bioactivities play in vivo. Here, we identified novel synaptic organizers in vitro then used a set of targeted mouse mutants to ask whether they participate in patterning a single synapse, the NMJ. Our results, summarized in Figure 7, lead to three main conclusions. First, at least three sets of presynaptic organizers (FGFs, β2-laminins, and collagens IV) are required for complete presynaptic differentiation at the NMJ. Second, these organizers act sequentially to pattern the motor nerve terminal. Third, part of the explanation for the sequential effects of the FGF, laminin, and collagen signaling systems lies in their distinct developmental regulation.

Multiple Organizers of Motor Nerve Terminals

FGFs have been implicated in multiple aspects of neural development but have been little studied in the context of synaptogenesis. We isolated FGF22 in a search for brain-derived proteins capable of promoting presynaptic differentiation in cultured neurons and then found that its closest relatives, FGF7 and -10, shared this bioactivity (Umemori et al., 2004). FGF22 is synthesized by cerebellar granule cells and promotes differentiation of their main synaptic inputs, mossy fibers, in vivo. Because FGF7, -10, and -22 are all expressed by developing muscle, we

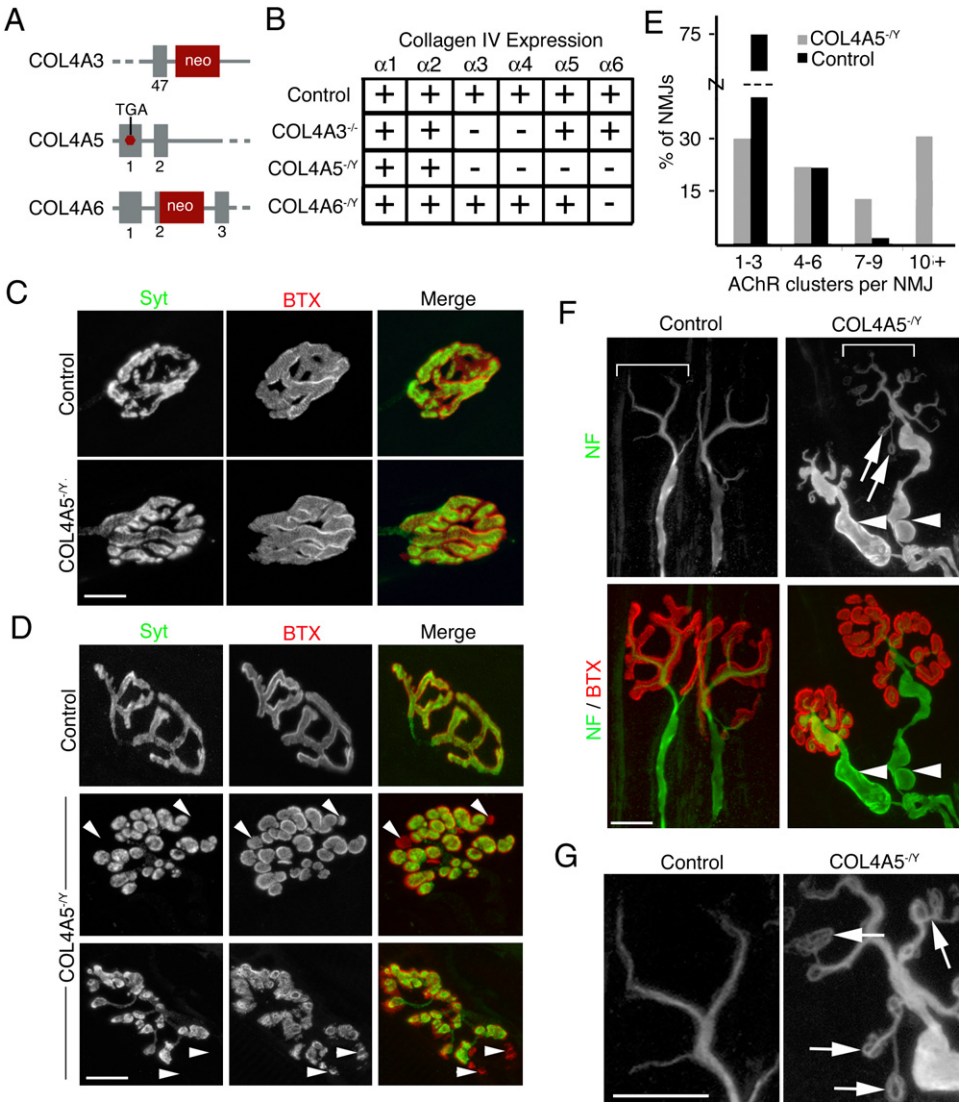

Figure 6. Collagen IV-Dependent Maintenance of the NMJ

(A) Schematic representation of COL4A3, COL4A5, and COL4A6 mutants. For COL4A3$^{-/-}$ mutants, the first three exons in the NC1 domain were replaced with a neo cassette. For COL4A5$^{-/Y}$ mutants, a point mutation converting a glycine to a STOP was inserted in exon 1. For COL4A6$^{-/Y}$ mutants, parts of exon 2 and intron 2 were replaced with a neo cassette. All three mutants are functional nulls.

(B) Collagen α(IV) chain content of synaptic cleft at the NMJ in wild-type and mutant mice, as predicted by protomer composition (B) and confirmed by immunofluorescence (Figure S4).

(C) NMJs from COL4A5$^{-/Y}$ and littermate controls at P21 stained with antibodies to synaptotagmin 2 (Syt) plus BTX. No defects are detectable in mutants during the first 3 postnatal weeks.

(D) NMJs from P56 COL4A5$^{-/Y}$ and littermate control diaphragm, stained with antibodies to synaptotagmin 2 (Syt) and BTX. Receptors are fragmented rather than branched in mutants, and some AChR-rich patches (arrowheads) are unapposed by presynaptic boutons.

(E) Number of discrete AChR fragments per NMJ in COL4A5$^{-/Y}$ and control muscles (n = 100 in mutant and 80 in controls NMJs).

(F) NMJs from P56 COL4A5$^{-/Y}$ and littermate control diaphragm stained with antibodies to neurofilaments (NF) plus BTX. Portions of preterminal axons are distended in mutants (arrowheads), and neurofilaments terminate in whorls in some mutant terminals (arrows). Brackets indicate areas enlarged in (G).

(G) Higher magnification of neurofilament in controls and COL4A5$^{-/Y}$ NMJs. Arrows highlight neurofilaments terminating in whorls in mutant terminals. Bar is 5 μm in (C), (D), (F), and (G).

used isoform-specific and conditional alleles of their common receptor, FGFR2, to probe their role. Results from FGFR2b$^{-/-}$ mice are subject to the caveat that the mutation affects all cells, and results from FGFR2$^{flox/flox}$ mice are subject to the caveat that both FGFR2 isoforms were deleted, but the congruence of results from the two alleles gives us confidence that FGF7/10/22 signaling to motor axons is required for complete presynaptic differentiation.

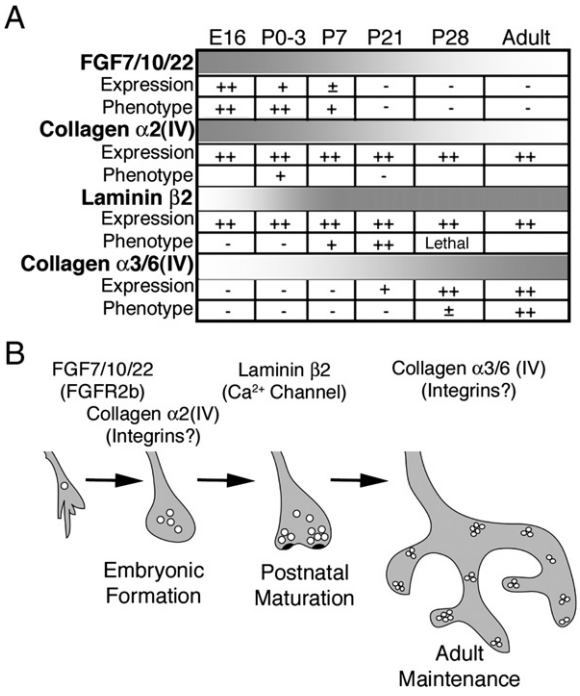

Figure 7. Sequential Effects and Expression of Multiple Presynaptic Organizers

(A) Neuromuscular expression of FGF7/10/22, β2 laminins, and collagen IV chains along with defects resulting from their loss.

(B) Model of sequential synaptic organizers. FGFs of the 7/10/22 subfamily act through FGFR2b to cluster synaptic vesicles in embryos. Collagen α2(IV) also promotes clustering at early stages. β2 laminins act through calcium channels to promote postnatal maturation of nerve terminals. Collagens α3 and α6 (IV) are required for synaptic maintenance in adults.

In fact, we may have underestimated their role in that members of this subfamily activate FGFR1b to a minor extent under some circumstances (Zhang et al., 2006).

A second organizer, laminin β2, was initially identified as a component of the synaptic cleft at the NMJ. In vitro and in vivo studies demonstrated a role for muscle-derived β2 laminins in the differentiation of motor nerve terminals (Hunter et al., 1989; Noakes et al., 1995; Son et al., 1999; Knight et al., 2003; Miner et al., 2006). Integrins and dystroglycan are the predominant cell-surface receptors for laminins in many cell types, but at the NMJ laminin β2 appears to act by binding to and clustering voltage-sensitive calcium channels, which then recruit and stabilize other components of the release apparatus (Nishimune et al., 2004).

The third organizers, the collagen IV chains, have not hitherto been implicated in synaptogenesis. Recently, the NC1 domains of basement membrane collagens IV, XV, and XVIII have been found to possess a variety of bioactivities in vitro, including modulation of angiogenesis and adhesion (reviewed in Ortega and Werb, 2002). Evidence for roles in neural development includes the demonstration that the NC1 domain of collagen IV promotes neurite outgrowth from cultured rodent sympathetic neurons (Lein et al., 1991) and that the NC1 domain of collagen XVIII regulates axon guidance in *C. elegans* and zebrafish (Ackley et al., 2001; Schneider and Granato, 2006).

Our results suggest that collagen IV chains play two distinct roles in vivo. During early development, the collagen α2(IV) chains are necessary for the proper differentiation of motor nerve terminals. These collagen IV chains might compensate for the absence of FGF signaling and laminin β2 in mutants lacking both organizers (Figure 4). After nerve terminals have matured, the synapse-specific collagen α3 and α6(IV) chains are necessary to maintain terminals. Although postsynaptic fragmentation is observed in mature COL4A5$^{-/Y}$ NMJs, we believe that collagen IV affects nerve terminals directly because no postsynaptic defects were evident in neonatal COL4A1$^{\Delta ex40/+}$ mice, and presynaptic defects predominate in 1-month-old COL4A5$^{-/Y}$ mice.

Interestingly, whereas loss of other presynaptic organizers led to synaptic defects in most muscles, loss of collagens α3–6(IV) predominantly affected the diaphragm (data not shown). One possible explanation for this selectivity is that it results from intermuscular differences in the relative levels of the α1/2 versus the α3–6(IV) chains. It is worth noting that marked intermuscular differences in phenotype have been observed in mutants lacking another synaptic organizer, agrin (Pun et al., 2002).

Sequential Effects of Presynaptic Organizers

Genetic analysis demonstrated that FGFs, laminins, and collagens IV act sequentially at a single synapse: FGF7/10/22 and collagen α1/2(IV) in embryonic synapses, β2 laminins in early postnatal synapses, and collagens α3–6(IV) in mature synapses. Thus, one answer to a question that motivated this work—why so many organizers?—is that they act sequentially to pattern the NMJ by separately affecting its formation, maturation, and maintenance.

What determines the sequence in which FGFs, β2 laminins, and collagens IV act? One factor is their patterns of expression. Levels of FGF7, -10 and -22 mRNA in myotubes and of FGFR2 in nerve terminals decline dramatically shortly after birth, whereas, α3(IV) and α6(IV) chains of collagen IV become abundant only after the second postnatal week. Developmental regulation of laminin β2 and collagen α2(IV), in contrast, does not readily explain the timing of their effects: both are present in the synaptic cleft from prenatal stages into adulthood. Interestingly, however, the P/Q-type calcium channel (Cav2.1) replaces its homolog, the N-type channel (Cav2.2), in motor nerve terminals during the first postnatal week (Urbano et al., 2002). Since laminin β2 binds to and signals through calcium channels, it will be interesting to test whether it differentially binds to or signals through Cav2.1 and Cav2.2. Likewise, collagen α3–6(IV) may render the α2(IV) chain redundant postnatally.

Although we have focused on the temporal differences among organizers, it is also possible that each affects the formation and/or maintenance of distinct presynaptic

structures. Differentiation of the nerve terminal involves multiple steps, including formation of active zones, clustering of vesicles, recruitment of calcium channels, acquisition of release capabilities, aggregation of mitochondria, and so on. Triggering any of these steps may lead to assembly of a nerve terminal in vitro, but each might be regulated separately in vivo. Indeed, each presynaptic organizer appears to have a different receptor on the motor nerve terminal. Thus, each target-derived organizer may have a different primary effect on a different aspect of presynaptic assembly.

How Many Organizers?

Each of the organizers we have studied is part of a group. Three FGFs (FGF7, FGF10, and FGF22) that signal through FGFR2b and promote vesicle clustering in vitro are expressed by myotubes. Three laminin heterotrimers containing the β2 subunit (α2β2γ1, α4β2γ1, and α5β2γ1) are assembled by muscle and are present in the synaptic cleft at the NMJ (Patton et al., 1997). Three collagen α(IV) chains capable of promoting vesicle clustering in vitro (α2, α3, and α6) are also present in the synaptic cleft. Thus, the potential exists for complex and subtle regulation of presynaptic differentiation.

However, even more presynaptic organizers may be involved in patterning the NMJ. Even in the absence of both laminin β2 and FGFR2, considerable presynaptic differentiation occurs at sites of nerve-muscle contact, and animals are viable for at least 2 postnatal weeks. As noted above, these additional factors might include FGFs signaling through FGFR1b and collagen α2(IV). Additional candidates include signals that have been shown to act on central neurons, such as SynCAM, eph kinases/ephrins, neuroligin/neurexin, and Wnts (see Introduction). Studies of Wnts in *Drosophila* (Packard et al., 2002) and of ephrins (Feng et al., 2000) and neurexins (Sons et al., 2006) in mouse are consistent with—but do not, so far, provide direct evidence for—this idea. Alternatively, additional organizers may remain to be discovered. Indeed, FGFs and collagen IV account for only part of the presynaptic organizing activity we have detected in brain (Umemori et al., 2004) and *Torpedo* (this study), respectively.

EXPERIMENTAL PROCEDURES

Cell Culture

Motoneurons were purified from E5.5 chick embryos and cultured as previously described (Henderson et al., 1996; Umemori et al., 2004). *Torpedo* extracts were added to cultures 4–8 hr after plating. Human recombinant collagen IV NC1 domains were prepared as previously described (Petitclerc et al., 2000) and added to cultures >5 hr after plating. After 48 hr in culture, motoneurons were fixed and stained with anti-synapsin, and vesicle-rich puncta were counted on an epifluorescence microscope (Zeiss). For RT-PCR, total RNA was prepared from cultured cells using an RNA purification kit (Stratagene).

C2 cells (ATCC, Manassas, VA) were cultured as previously described by Kummer et al. (2004). For studies of AChR clustering, recombinant NC1 domains (5 μg/ml) and/or agrin (1 μM) were added with fusion medium. For cocultures, C2 myoblasts were plated in 8-well Lab-Tek Permanox chamber slides, grown until confluent, and fused in medium containing 2% horse serum. Two days later, 2000 motoneurons per well were added and maintained in motoneuron medium. FGFR2bAP or FGFR2cAP were prepared as described by Umemori et al. (2004) and added with motoneurons. Three days later, cultures were fixed and stained as described below.

Animals

A naturally occurring COL4A1 mutant in which the fortieth exon is deleted was described previously (COL4A1$^{\Delta ex40}$; Gould et al., 2005, 2006). Two copies of COL4A1$^{\Delta ex40}$ act as a functional null, thereby terminating embryogenesis before any synapses form. However, a single copy of COL4A1$^{\Delta ex40}$ reduces the amount of collagen secreted and assembled into the basal lamina. The COL4A1$^{\Delta ex40}$ mutation was backcrossed into two different strains (15 generations onto C57/B6 and 5 generations onto BALB/C); the synaptic phenotype at P3 was observed in both genotypes. Targeted null mutants of the collagen α3(IV) gene (COL4A3$^{-/-}$; Miner and Sanes, 1996), the collagen α5(IV) gene (COL4A5$^{-/Y}$; Rheault et al., 2004, and the laminin β2 gene (LAMB2$^{-/-}$; Noakes et al., 1995) were described previously. All have been shown to be functional nulls. Two alleles of the FGFR2 gene were also described previously: a conditional allele in which Cre-mediated excision leads to a functional null (FGFR2$^{flox/flox}$; Yu et al., 2003) and one in which a nonsense mutation in the unique exon of the FGFR2b isoform leads to generation of a truncated nonfunctional protein without affecting expression of FGFR2c (FGFR2b$^{-/-}$; Eswarakumar et al., 2002). A neomycin resistance cassette flanked by loxP sites was present in the FGFR2b- and COL4A5-targeting vectors, but in both cases they were removed by Cre-mediated recombination before generating the mice used here. Transgenic mice in which expression of YFP is dependent on Cre-mediated excision (Thy1-Stop-YFP; Buffelli et al., 2003) were generated in our laboratory and are now available from Jackson Laboratories (Bar Harbor, ME). Transgenic mice in which Cre recombinase is expressed under control of regulatory elements from the HB9 gene (HB9-Cre) or the Islet1 gene (Isl-Cre; Srinivas et al., 2001) were used. To generate HB9-Cre mice, a previously characterized 10 kb genomic fragment (Lee et al., 2004) was placed upstream of a cassette containing a Cre cDNA and a polyadenylation signal from SV40; the insert was linearized, purified, and injected into pronuclei by standard techniques. All mutant and transgenic mice were maintained on a C57B6 background, and phenotypes of mutants were compared to those of littermate controls.

To generate a null mutant of the collagen α6(IV) gene (COL4A6$^{-/Y}$) parts of exon 2 and intron 2 were replaced with a neomycin resistance cassette. The mutated exon 2 lacked the signal peptide. Homologous recombinants were identified by Southern blot analysis using a 300 bp EcoRI-PstI external probe. Correctly targeted ES cells were injected into blastocysts to generate chimeras. Male chimeras were used to establish 129Sv inbred lines.

Histology

Whole mounts and cryostat sections of muscles were fixed and stained as described by Misgeld et al. (2002). For collagen staining, sections were fixed for 10 min in ice-cold acetone, treated with a 1:1 mixture of 0.1 M KCl and 0.1 N HCl, then washed in PBS. Mutant and control tissues were processed together, and images were acquired with identical settings on a CCD camera (Photometrics) and analyzed with Metamorph imaging software (Universal Imaging).

Sources of antibodies were as follows: anti-neurofilament, Covance Research Products (Denver, PA); anti-synaptophysin, Invitrogen (Carlsbad, CA); anti-FGFR2, Santa Cruz (Santa Cruz, CA) and Abcam Inc. (Cambridge, MA); anti-acetylated tubulin, Sigma (St. Louis, MO); anti-SV2, Developmental Studies Hybridoma Bank (University of Iowa; Iowa City, IA); anti-synaptotagmin 2 (znp-1), Zebrafish International Resource Center (Eugene, OR); and anti-entactin (nidogen-1), Chemicon (Temecula, CA). Rat monoclonal antibodies to collagen α1–6(IV) were described previously (Ninomiya et al., 1995). Anti-synapsin was a gift from P. Greengard and A. Nairn (Rockefeller University),

and anti-laminin β2 was a gift from R. Timpl (Max Planck Institute, Munich). A rat monoclonal antibody to several collagen IV NC1 domains will be described elsewhere (D.B. Borza, personal communication).

Purification of Synaptic Organizing Molecules

Electric organs from *Torpedo californica* were homogenized in 400 mM NaCl, 1 mM EDTA, and 1 mM EGTA in 10 mM Tris (pH 7.5) containing 0.1 mg/ml PMSF (TP), then spun at 17,700 g for 30 min. The pellet and loosely associated fibrous layer were homogenized in 150 mM NaCl, 1 mM EDTA, and 1 mM EGTA in TP, then spun at 17,700 g for 30 min. Salts were removed by homogenizing the pelleted material in TP. After centrifugation at 17,700 g for 30 min, loosely associated fibrous material was removed from the pellet by resuspending and stirring in TP with 3% (w/v) TritonX100 for 30 min. After centrifugation at 30,100 g, the pellet was again resuspended and stirred in TP with 3% TritonX100 for 30 min. Insoluble material was pelleted by centrifugation; washed with TP; homogenized in 0.2 M sodium bicarbonate (pH 9.0), 5% glycerol, 1 M NaCl, and 0.1 mg/ml PMSF (SB); then stirred overnight. Subsequently, SB containing insoluble material underwent six cycles of sonication (10 min), homogenization (10 min), and stirring (30 min) then was centrifuged at 30,100 g for 30 min. The supernatant was dialyzed against 10 mM Tris (pH 8.0) and 25 mM NaCl (buffer A), applied to a DEAE-Sepharose FF column, and eluted with a 0–300 mM linear NaCl gradient in buffer A (AKTAprime FPLC System; Pharmacia). Active fractions were pooled, dialyzed against buffer A, applied to a 20 ml HiTrap Q column, and eluted with a 0–500 mM linear NaCl gradient. Active fractions were pooled again, concentrated to 500 μl by centrifugal filtration (Amicon Ultra, Millipore), applied to a Superdex-200 HR10/30 gel filtration column, and separated in buffer A with 150 mM NaCl. Active fractions were pooled, dialyzed against 20 mM sodium acetate buffer (pH 5.0), applied to a MONO-S HR 5/5 column, and eluted with a 0–500 mM NaCl linear gradient. All steps were performed at 4°C. Concentrated active fractions were separated on a 4%–20% gradient SDS-polyacrylamide Ready-Gel (BioRad; Hercules, CA) and stained with Colloidal Coomassie Blue (Invitrogen). Bands were excised for mass spectrometric analysis at the Harvard University Microchemistry & Proteomics Analysis Facility. For removal of collagenous sequences, active fractions were incubated with 100 or 1000 Units of ultrapure collagenase (Sigma, St.Louis, MO) for 4 hr at 37°C.

Supplemental Data

Supplemental Data include eight figures and two tables and can be found with this article online at http://www.cell.com/cgi/content/129/1/179/DC1/.

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