ERRATUM

Restoration of synapse formation in *Musk* mutant mice expressing a Musk/Trk chimeric receptor

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On page 5454 of this article, the first paragraph in the section 'Motor axons extend…' should read 'Based on the expression of transgenes containing the MCK enhancer and promoter, the endogenous *MCK* gene is activated in skeletal muscle at **~E13.5** (S. Hauschka, personal communication), 1 day after motor axons first enter the muscle.'

We apologise to the authors and readers for this mistake.

Restoration of synapse formation in *Musk* mutant mice expressing a Musk/Trk chimeric receptor

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SUMMARY

Mice lacking Musk, a muscle-specific receptor tyrosine kinase that is activated by agrin, fail to form neuromuscular synapses and consequently die at birth because of their failure to move or breathe. We produced mice that express a chimeric receptor, containing the juxtamembrane region of Musk and the kinase domain of TrkA, selectively in muscle, and we crossed this transgene into *Musk* mutant mice. Expression of this chimeric receptor restores presynaptic and postsynaptic differentiation, including the formation of nerve terminal arbors, synapse-specific transcription, and clustering of postsynaptic proteins, allowing *Musk* mutant mice to move, breathe and survive as adults. These results show that the juxtamembrane region of Musk, including a single phosphotyrosine docking site, even in the context of a different kinase domain, is sufficient to activate the multiple pathways leading to presynaptic and postsynaptic differentiation in vivo. In addition, we find that Musk protein can be clustered at synaptic sites, even if *Musk* mRNA is expressed uniformly in muscle. Moreover, acetylcholine receptor clustering and motor terminal branching are restored in parallel, indicating that the extent of presynaptic differentiation is matched to the extent of postsynaptic differentiation.

Supplemental data available on-line

Key words: Agrin, Acetylcholine receptor, Synapse-specific transcription, Receptor tyrosine kinase, Neuromuscular synapse, Mouse

INTRODUCTION

Neuromuscular synapses form following a series of complex interactions between motor neurons, muscle fibers and Schwann cells (Burden, 1998; Sanes and Lichtman, 1999; Schaeffer et al., 2001; Son and Thompson, 1995). Agrin, a 200 kDa protein synthesized by motor neurons, is a critical synaptic signaling molecule that organizes postsynaptic differentiation by stimulating Musk, a receptor tyrosine kinase (RTK) expressed selectively in skeletal muscle (Glass and Yancopoulos, 1997; McMahan, 1990). Embryos lacking agrin or Musk fail to form neuromuscular synapses and consequently die at birth due to their failure to move or breathe (DeChiara et al., 1996; Gautam et al., 1996). Neurogenesis and myogenesis appear normal in *agrin* and *Musk* mutant embryos, but skeletal muscle-derived proteins, including acetylcholine receptors (AChRs), which are normally concentrated at synaptic sites, are instead expressed uniformly in muscle of *Musk* mutant mice. In addition, AChR genes, which are transcribed selectively in synaptic nuclei of wild-type mice, are expressed throughout the myofiber of *Musk* mutant mice. Presynaptic differentiation is also aberrant in *agrin* and *Musk* mutant mice, as motor axons fail to stop or differentiate and instead wander throughout the muscle. Taken together with experiments showing that Musk

is activated by agrin, these results indicate that agrin stimulation of Musk leads to clustering of critical muscle-derived proteins, including AChRs, activation of synapse-specific gene expression and the induction and/or reorganization of a retrograde signal for presynaptic differentiation (DeChiara et al., 1996; Gautam et al., 1996; Glass et al., 1996).

Domains in Musk that are important for Musk signaling have been defined by introducing wild-type or mutant forms of Musk into *Musk* mutant muscle cell lines (Herbst and Burden, 2000; Zhou et al., 1999). These studies have revealed an essential role for one of the two juxtamembrane tyrosine residues in Musk (Y553), as mutation of this tyrosine abrogates the ability of agrin to induce clustering or tyrosine phosphorylation of AChRs in cultured myotubes. This tyrosine appears to have a dual function in Musk signaling, as this tyrosine is required both to activate Musk kinase activity fully and to recruit a signaling component(s) that functions downstream from Musk (Herbst and Burden, 2000). Evidence for such a dual role is based upon analysis of Musk/TrkA chimeric receptors. Agrin stimulates tyrosine phosphorylation of a chimeric receptor composed of the extracellular and transmembrane regions of Musk, and the intracellular region of TrkA (Nrtk1 – Mouse Genome Informatics), but phosphorylation of this chimeric receptor fails to induce

AChR clustering or tyrosine phosphorylation in muscle cell lines. The same Musk/TrkA chimera, but including a substitution of thirteen amino acids from the juxtamembrane region of Musk, including Y553, with the comparable region in TrkA is similarly tyrosine phosphorylated by agrin stimulation, but tyrosine residue phosphorylation of this chimera leads to clustering and tyrosine phosphorylation of AChRs in cultured myotubes (Herbst and Burden, 2000). These experiments indicate that the juxtamembrane region of Musk, even in the context of a different kinase domain, is sufficient to activate a signaling pathway leading to the clustering and tyrosine phosphorylation of AChRs in muscle cell lines. Nonetheless, as cultured muscle cells are not amenable to study all aspects of synaptic differentiation, these experiments could not address whether this juxtamembrane region of Musk is sufficient to confer additional aspects of Musk signaling, including clustering of additional postsynaptic proteins, synapse-specific transcription, presynaptic differentiation and synapse formation. Moreover, requirements for synaptic proteins may differ in cell culture and in vivo; for example, rapsyn is required to cluster Musk in cultured cells but not at synapses in vivo (Gillespie et al., 1996; Moscoso et al., 1995), and the ectodomain of Musk is required to cluster AChRs in cultured cells but not in vivo (Apel et al., 1997; Sander et al., 2001). Thus, in vivo studies are required to delineate the signaling mechanisms that lead to the complex biological response initiated by Musk at synapses.

In addition to Y553 in the juxtamembrane region of Musk, agrin stimulates the phosphorylation of five tyrosine residues in the kinase domain of Musk (Watty et al., 2000). In other RTKs, recruitment of different adaptor proteins to distinct phosphotyrosine docking sites leads to activation of disparate signaling pathways, which are often coupled to different biological responses (Madhani, 2001; Pawson and Scott, 1997). For example, in TrkA, the receptor for nerve growth factor, phosphorylation of a juxtamembrane tyrosine leads to Ras and PI3-kinase activation, which are important for cell survival and neurite outgrowth (Greene and Kaplan, 1995; Huang and Reichardt, 2001), whereas phosphorylation of a tyrosine in the C-terminal region of TrkA leads to PLCγ activation, which is crucial for NGF-dependent Na^+ channel and VRI channel regulation (Choi et al., 2001; Chuang et al., 2001). Moreover, in TrkB, phosphorylation of a single juxtamembrane tyrosine is required for nearly all of NT4-dependent signaling whereas phosphorylation of other tyrosine residues are required to mediate BDNF-dependent signaling in vivo (Minichiello et al., 1998). Similarly, in Met, the receptor for hepatocyte growth factor, a binding site for Grb2 is essential for late steps in myogenesis (Maina et al., 1996), a binding-site for PI3-kinase is essential for placental development, hepatocyte survival and myoblast migration, and a Src binding-site is essential for motor axon outgrowth (Maina et al., 2001). To determine whether clustering of postsynaptic proteins, synapse-specific transcription and presynaptic differentiation depend upon multiple, distinct docking sites in Musk, we produced mice that expressed the Musk/TrkA chimera, containing thirteen amino acids from the juxtamembrane region of Musk and the kinase domain of TrkA, and we crossed this transgene into Musk mutant mice. We found that expression of this chimeric receptor

could restore all aspects of postsynaptic and presynaptic differentiation that were defective in Musk mutant mice. Moreover, we found that accumulation of Musk protein at synaptic sites was not dependent upon synaptic localization of Musk mRNA. These results indicate that the juxtamembrane region of Musk, including a single phosphotyrosine docking site, even in the context of a different kinase domain, is sufficient to activate the multiple pathways that lead to presynaptic and postsynaptic differentiation in vivo.

MATERIALS AND METHODS

Generation of *MCK-Musk* and *MCK-MMT* transgenic mice

GFP was ligated in frame to the C terminus of the rat *Musk* cDNA using the pEGFP N1 vector (Clontech). Lithmus 29 (New England Biolabs) was modified by introducing a *Age*I/*Kpn*/*Sac*I/*Age*I cassette into the *Age*I site and removing the original *Kpn*I and *Sac*I sites from the polylinker; the MCK promoter/enhancer/intron cassette was excised from pBS-MCK (Bruning et al., 1998) and subcloned into the modified, *Kpn*I/*Sac*I-digested Litmus 29 plasmid. The *Musk-GFP* and the *MMT* (Herbst and Burden, 2000) constructs were subcloned into Litmus 29/MCK. Both the *Musk* and *MMT* constructs contain a FLAG epitope tag in the extracellular domain (Herbst and Burden, 2000). The *MCK-Musk* and *MCK-MMT* constructs were excised from Lithmus 29 using *Age*I and gel purified. DNA was injected into the male pronucleus of B6/D2 fertilized eggs, which were subsequently transferred into pseudopregnant foster mice; progeny were genotyped by Southern blotting and PCR, and transgene-containing founder mice were crossed to *Musk*$^{+/-}$ mice.

Mouse genotyping

Tail DNA of F$_1$ mice was digested with *Eco*RI and analyzed by Southern blotting using probes from the rat *Musk* (nucleotides 2080-2708) and rat *TrkA* (nucleotides 2191-2572) kinase domains (Meakin et al., 1992). Subsequent generations of mice were genotyped by PCR using primers that are specific for sequences in the extracellular region of the mouse *Musk* cDNA (5′-GAAGCAACCTTTCCTTCCTGAG-3′ and 5′-ATTTTCCCTGAGAGCATTGTCC-3′) using the following conditions: one cycle of 94°C for 2 minutes, 40 cycles of 94°C for 30 seconds, 58°C for 30 seconds and 72°C for 40 seconds followed by 1 cycle of 72°C for 2 minutes. The wild-type and mutant *Musk* alleles (DeChiara et al., 1996) were detected by PCR, using primers that are specific for sequences in the *Musk* kinase domain and the *neo* gene (5′-ATGCCGCCCGAGTCTATGTTCTAC-3′, 5′-TTCTCCTGGC-AAACAATCAACTGG-3′ and 5′-CATAGCCTGAAGAACGAGA-TCAGCAGC-3′), using the following conditions: one cycle of 94°C for 2 minutes, 40 cycles of 94°C for 30 seconds, 56°C for 30 seconds and 72°C for 40 seconds followed by one cycle of 72ºC for 2 minutes. We examined three *MCK-Musk* lines and two *MCK-MMT* lines; analyzed mice were heterozygous for *MCK-Musk* or *MCK-MMT*. We noted attrition in large litters up to the time of weaning, indicating that *Musk*$^{-/-}$; *MCK-MMT* and, in particular, *Musk*$^{-/-}$; *MCK-Musk* pups competed poorly with their wild-type littermates for nourishment. *Musk*$^{-/-}$; *MCK-MMT* and *Musk*$^{-/-}$; *MCK-Musk* mice are smaller than their littermates during this period, but attain the same weight as their littermates by one month after birth. We assessed presynaptic and postsynaptic differentiation in embryos and in 3-week postnatal mice, when neuromuscular synapses are fully mature. *Musk*$^{-/-}$; *MCK-Musk* and *Musk*$^{-/-}$; *MCK-MMT* adult mice appeared to have normal motility as they performed identically to wild-type mice on a Rotarod at a constant speed (32 rpm). Rescued adult mice show no signs of a shortened longevity, as they have been maintained for an excess of 1 year.

Immunohistochemistry

Diaphragm muscles were dissected from embryos or three week old mice, fixed for 90 minutes in 1% formaldehyde, rinsed in PBS and incubated with 0.1 M glycine in PBS for 15 minutes. After dissection of the overlying connective tissue, the muscles were permeabilized in 0.5% Triton X-100 in PBS (PBST) for 5 minutes, incubated overnight at 4°C with rabbit polyclonal antibodies against neurofilament (1:500, Chemicon) and synaptophysin (1:5, Zymed) in 4% goat serum/2% BSA/PBST, washed three times for 20 minutes in PBST, incubated overnight at 4°C with fluorescein-conjugated goat anti-rabbit IgG (1:200, Jackson Immunoresearch) and Texas Red-conjugated α-BGT (Molecular Probes). The muscles were washed twice for 20 minutes in PBST, twice for 20 minutes in PBS, postfixed in 1% formaldehyde for 10 minutes, rinsed in PBS, flat mounted in Vectashield (Vector Labs) and viewed with optics selective for either fluorescein or Texas Red.

Frozen sections (10 μm) from fixed, adult leg muscles were stained with antibodies as described previously (DeChiara et al., 1996; Zhu et al., 1995). The following primary antibodies were used: affinity-purified rabbit anti-rapsyn (1:500) (Herbst and Burden, 2000), mouse anti-utrophin C terminus (1:20, Vector Labs), rabbit anti-Na+ channel (1:1000, Upstate), rabbit anti-ErbB4 (antibody # 616) (Zhu et al., 1995), rabbit anti-synaptophysin (1:20, Zymed), mouse anti-SV2 (1:10) (Buckley and Kelly, 1985) and rabbit anti-Musk (#83033, 1:4000 and #24908, 1:50) (Watty et al., 2000). We were unable to detect fluorescence from the Musk-GFP transgene.

Whole-mount in situ hybridization

Intercostal muscles were fixed in 4% formaldehyde, dehydrated in methanol, digested with Proteinase K, probed with a digoxigenin-labeled riboprobe transcribed from an AChR α (DeChiara et al., 1996), AChR δ subunit (Simon et al., 1992) or a rat *Musk* cDNA (nucleotides 1-1663) and processed as described elsewhere (Wilkinson, 1992). A low level of uniform staining was observed with control, sense probes for the AChR α and δ subunits.

Immunoprecipitation and western blotting

Tissues were homogenized and lysed as described previously (Bruning et al., 1998). Tissues were homogenized in lysis buffer

Fig. 1. *MCK-Musk* and *MCK-MMT* transgenes are expressed and active in skeletal muscle. (A) Expression of Musk and MMT are controlled by the 5′ regulatory region of the muscle creatine kinase (*MCK*) gene. *MCK-Musk* and *MCK-MMT* transgenes each contain a FLAG epitope-tag in the extracellular domain of Musk (Herbst and Burden, 2000). The *MCK-Musk* transgene encodes a fusion protein between Musk and GFP. MMT, a chimera between the extracellular and transmembrane regions of Musk and the intracellular region of TrkA, includes a substitution of 13 amino acids from the juxtamembrane (JM) of Musk with the comparable region in TrkA (Herbst and Burden, 2000). (B) Transgene expression was measured by immunoprecipitation followed by western blotting. MCK-Musk and MCK-MMT are expressed strongly in skeletal muscle; heart expression is detected in some, but not all lines (arrows). TrkA is expressed strongly in brain. (C) Transgene expression, determined by western blotting of skeletal muscle lysates, differs among the different transgenic (Tg) lines: *MCK-MMT*, line 5; expresses more MMT in skeletal muscle than *MCK-MMT* (line 29), and *MCK-Musk* (line 23) expresses more Musk in skeletal muscle than *MCK-Musk*, line 47. (D) Musk as well as MMT are tyrosine phosphorylated. Musk and MMT were immunoprecipitated from skeletal muscle with antibodies to Musk and antibodies to TrkA, respectively, and western blots were probed with antibodies to phosphotyrosine (pY) and FLAG.
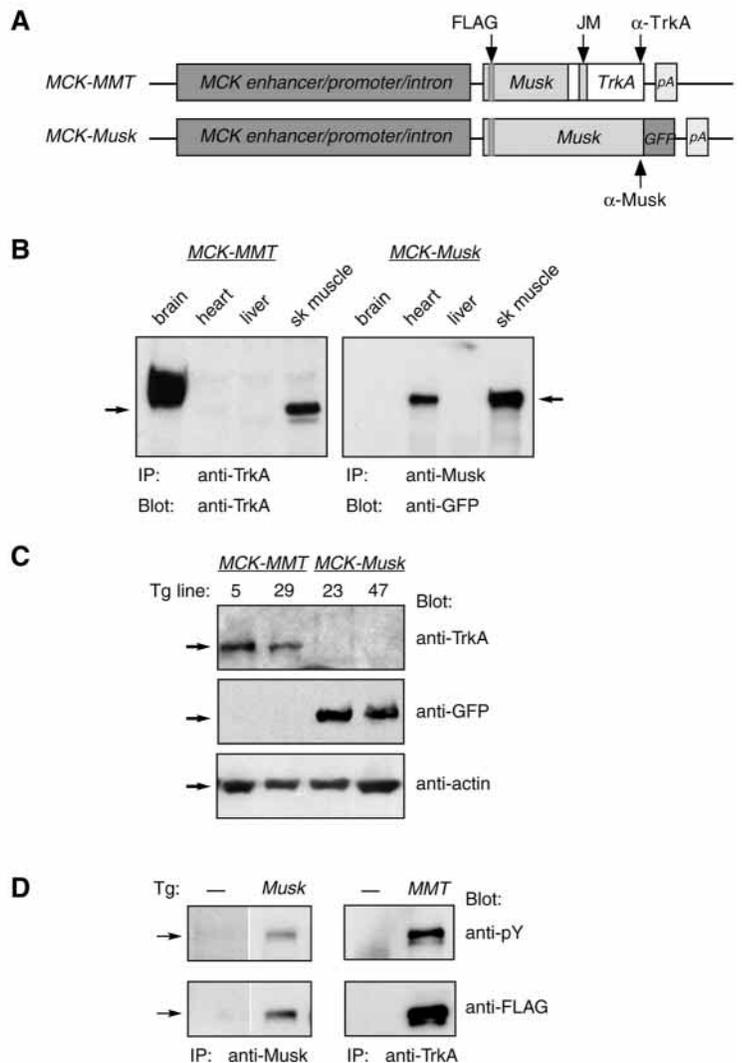
[50 mM HEPES (pH 7.4), 50 mM sodium pyrophosphate, 0.1 M sodium fluoride, 10 mM EDTA, 10 mM sodium orthovanadate, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 2 mM PMSF]. After addition of an equal volume of lysis buffer containing 2% Triton X-100, the lysates were incubated at 4°C for 30 minutes and pre-cleared by centrifugation (20 minutes at 100,000 *g* in a TLA 100.3 rotor in a TL-100 ultracentrifuge) (Beckman). Proteins were immunoprecipitated and analyzed by western blotting as described previously (Herbst and Burden, 2000).

Quantitation of AChR density and synaptic area

The density of AChRs at synapses in diaphragm muscles from 3-week-old mice was quantitated from data captured with a Zeiss 510 confocal microscope using a 3D software program provided by the manufacturer. In each experiment, diaphragms from wild-type and mutant mice were stained together. While viewing a wild-type muscle, the gain of the amplifier was adjusted to a subsaturating level, and this setting was maintained while viewing mutant muscles. We examined at least three mice from each genotype, and images from at least ten synapses in each muscle were included in the analysis.

Quantitation of axon growth

Diaphragm muscles were stained with antibodies to NF and synaptophysin. Images were captured on a CCD camera (Princeton Instruments), attached to a Zeiss Axioskop and analyzed using

Table 1. *MCK-Musk* and *MCK-MMT* rescue *Musk* mutant mice

	MCK-MMT (#29)			MCK-MMT (#5)			MCK-MusK (#23)		
	Observed	Expected	χ-square	Observed	Expected	χ-square	Observed	Expected	χ-square
$Musk^{+/-}$; MCK-Tg	7	11.7	1.9	12	10.6	0.18	8	7.1	0.11
$Musk^{+/-}$	13	11.7	0.14	12	10.6	0.18	8	7.1	0.11
$Musk^{+/+}$; MCK-Tg	7	5.8	0.25	3	5.3	1	2	3.6	0.7
$Musk^{+/+}$	11	5.8	4.7	8	5.3	1.37	6	3.6	1.6
$Musk^{-/-}$; MCK-Tg	3	5.8	1.35	2	5.3	2.05	1	3.6	1.9
$Musk^{-/-}$	0	0	0	0	0	0	0	0	0
Total	41		8.34	37		4.78	25		4.4
P			0.15			0.45			0.5
$Musk^{+/+}$ or $Musk^{+/-}$; MCK-Tg	14	17.5	0.7	15	15.9	0.05	10	10.7	0.05
$Musk^{+/+}$ or $Musk^{+/-}$	24	17.5	2.4	20	15.9	1.06	14	10.7	1.02
Total	38		3.1	35		1.11	24		1.07
P			0.08			0.3			0.3
$Musk^{+/-}$; MCK-Tg	7	11.7	1.9						
$Musk^{+/+}$; MCK-Tg	7	5.8	0.25						
$Musk^{-/-}$; MCK-Tg	3	5.8	1.35						
Total	17		3.5						
P			0.18						

Musk$^{+/-}$ mice were crossed with *Musk*$^{+/-}$; *MCK-Musk* or *Musk*$^{+/-}$; *MCK-MMT* mice, and we determined the genotype of the surviving progeny at P0 or P1. χ-square analysis of progeny from a cross of *Musk*$^{+/-}$ × *Musk*$^{+/-}$; *MCK-Musk* (line #23) mice or *Musk*$^{+/-}$ × *Musk*$^{+/-}$; *MCK-MMT* (line #5, line #29) mice shows that the occurrence of genotypes is unlikely ($P>0.05$) to occur by chance, indicating that the neonatal lethality of *Musk* mutant mice can be fully rescued by expression of *MCK-Musk* or *MCK-MMT*.

Metamorph. We measured the total number of pixels in two thresholded areas of the left hemi-diaphragm muscle, each adjacent to the main intramuscular nerve. The value in the first area, which is immediately adjacent to the main intramuscular nerve, provides an estimate of the number of axons that branched from the main nerve, while the value in the second area, which is adjacent to the main nerve but extends further toward the edge of the muscle, provides an estimate of the extent of axon growth away from the main intramuscular nerve. The value in each area of wild-type muscle was assigned as 100%, and the values in mutant muscles were expressed relative to wild type. By late embryonic stages, many motor axons in *Musk* mutant mice have reached the edge of the muscle; owing to the irregular shape of the muscle, however, we measured axon outgrowth in an area that did not extend to the edge of the muscle. Consequently, our procedure underestimates the increase in motor axon outgrowth in *Musk* mutant mice. Images from at least three diaphragm muscles from each genotype, at three different stages (E14.5, E16.5 and E18.5), were included in the analysis. We found that the number of axons that branch from the main intramuscular nerve was similar in wild-type and mutant embryos.

Antibodies

Antibodies (#83033) to Musk were produced by immunizing rabbits with a conjugate between KLH and an extracellular sequence (SCGALQVKMKPKITRPPINV) in Musk (Research Genetics). Antibodies to rapsyn were produced and purified as described previously (Herbst and Burden, 2000). The monoclonal antibody (M2) to the FLAG epitope was purchased from Sigma. Polyclonal antibodies to TrkA (C14) were purchased from Santa Cruz. The monoclonal antibody to phosphotyrosine (mAB 4G10) was purchased from Upstate. The monoclonal antibody to GFP was purchased from Clontech.

RESULTS

A Musk/TrkA chimera can substitute for endogenous Musk in vivo

To determine whether the Musk/TrkA chimera, including the juxtamembrane region of Musk, contains sequences that are sufficient for presynaptic and postsynaptic differentiation, we produced transgenic mice that express this chimera (MMT) under the control of the muscle-specific creatine kinase [MCK (*Ckmm* – Mouse Genome Informatics)] 5′ regulatory region (Fig. 1), and we subsequently crossed this transgene into *Musk* mutant mice.

We produced and analyzed two *MCK-MMT* transgenic lines, as well as three *MCK-Musk* control transgenic lines. These mice express the transgenes in a muscle-specific manner, as assessed by western blotting (Fig. 1), and the protein encoded by each transgene is tyrosine phosphorylated (Fig. 1). *MCK-MMT* and *MCK-Musk* transgenic mice were crossed to *Musk*$^{+/-}$ mice, and progeny were crossed to *Musk*$^{+/-}$ mice to generate mice that lack endogenous Musk but express either *MCK-MMT* or *MCK-Musk*. Unlike *Musk* mutant mice, which lack neuromuscular synapses and die at birth (DeChiara et al., 1996), *Musk* mutant mice expressing either *MCK-MMT* or *MCK-Musk* move, breathe and survive after birth (Table 1).

We stained wholemounts of diaphragm muscle from E18.5 mice with a mixture of antibodies to neurofilament and synaptophysin, to label axons and terminals, respectively, and with Texas Red α-Bungarotoxin (TR α-BGT) to label acetyl choline receptors (AChRs). We found that nerve terminals differentiate and that AChRs cluster in the rescued newborn mice (Fig. 2A). Moreover, synapses are maintained and continue to mature postnatally (Fig. 2B). These experiments demonstrate that the juxtamembrane region of Musk, even in the context of a kinase domain from a different tyrosine kinase, is sufficient to activate a signaling cascade that leads to clustering of AChRs in vivo. Furthermore, as MMT expression, selectively in muscle, restores presynaptic differentiation, these results indicate that the juxtamembrane region of Musk has a central role in producing and/or organizing retrograde signals required for presynaptic differentiation and synapse formation.

The density of AChRs at synaptic sites in rescued mice

We measured the density of AChRs at synaptic sites (Fig. 2B) and found that the density of synaptic AChRs is modestly lower in $Musk^{-/-}$; $MCK\text{-}Musk$ mice than in wild-type mice (line #23, 79±3.2%, n=3; line #54, 77%, n=1). These results indicate that the MCK regulatory region confers sufficient Musk expression to restore AChR clustering at synapses but that the level and/or pattern of $MCK\text{-}Musk$ expression differs from wild-type $Musk$ expression, resulting in a 20% reduction in AChR density at synapses. In $Musk^{-/-}$; $MCK\text{-}MMT$ mice, AChRs are likewise clustered at synapses, but at a reduced density (line #5, 66±3.4%, n=3; line #29, 48±0.8%, n=3). The lower density of synaptic AChRs in rescued mice expressing MMT may be due to a modestly (~twofold) lower expression of the MMT transgene (see supplementary data at http://dev.biologists.org/supplemental/) or to sequences in Musk that are absent from MMT. Nonetheless, the MMT chimera also restores AChR clustering at synapses.

MMT chimera contains sequences that are sufficient for clustering postsynaptic proteins

Agrin/Musk signaling is necessary and sufficient to cluster several muscle-derived proteins in addition to AChRs (DeChiara et al., 1996; Gautam et al., 1996; Meier et al., 1997; Rimer et al., 1998). We therefore examined whether the MMT chimera contains sequences sufficient for clustering these proteins at synaptic sites. We stained frozen sections of muscle from three week old mice with antibodies to: (1) rapsyn, an intracellular, peripheral membrane protein associated with AChRs; (2) utrophin, a component of the dystrophin-associated glycoprotein complex; (3) ErbB4, a receptor for neuregulin; and (4) Na^+ channels (Sanes and Lichtman, 1999). Fig. 3 shows that rapsyn, ErbB4, utrophin and Na^+ channels are each concentrated at synapses in $Musk^{-/-}$; $MCK\text{-}MMT$ mice. These results indicate that the MMT chimera contains sequences that are sufficient to restore the clustering of most, if not all postsynaptic proteins.

MMT chimera induces extrasynaptic AChR clusters

In innervated muscle fibers from wild-type and $Musk^{-/-}$; $MCK\text{-}Musk$ mice, AChR clusters are restricted to synaptic sites. In $Musk^{-/-}$; $MCK\text{-}MMT$ mice, however, AChR clusters are readily evident at ectopic sites in addition to neuromuscular synapses (Fig. 4). Because these ectopic AChR clusters are found on most muscle fibers and are often located immediately adjacent to synaptic sites, marked by terminal arbors and synaptic AChR clusters, these ectopic AChR clusters are not associated with muscle denervation. Moreover, other synaptic proteins, including rapsyn, ErbB4, utrophin and Na^+ channels, are each co-clustered with ectopic AChR clusters in $Musk^{-/-}$; $MCK\text{-}MMT$ mice (Fig. 4).

Synapse-specific transcription is activated by MMT

Agrin/Musk signaling is necessary to activate synapse-specific transcription, leading to an enrichment of AChR mRNA in the central, synapse-rich region of muscle (DeChiara et al., 1996; Gautam et al., 1996). We therefore used in-situ hybridization to determine whether AChR genes are expressed selectively in synaptic nuclei of $Musk^{-/-}$; $MCK\text{-}MMT$ mice. Fig. 5 shows that
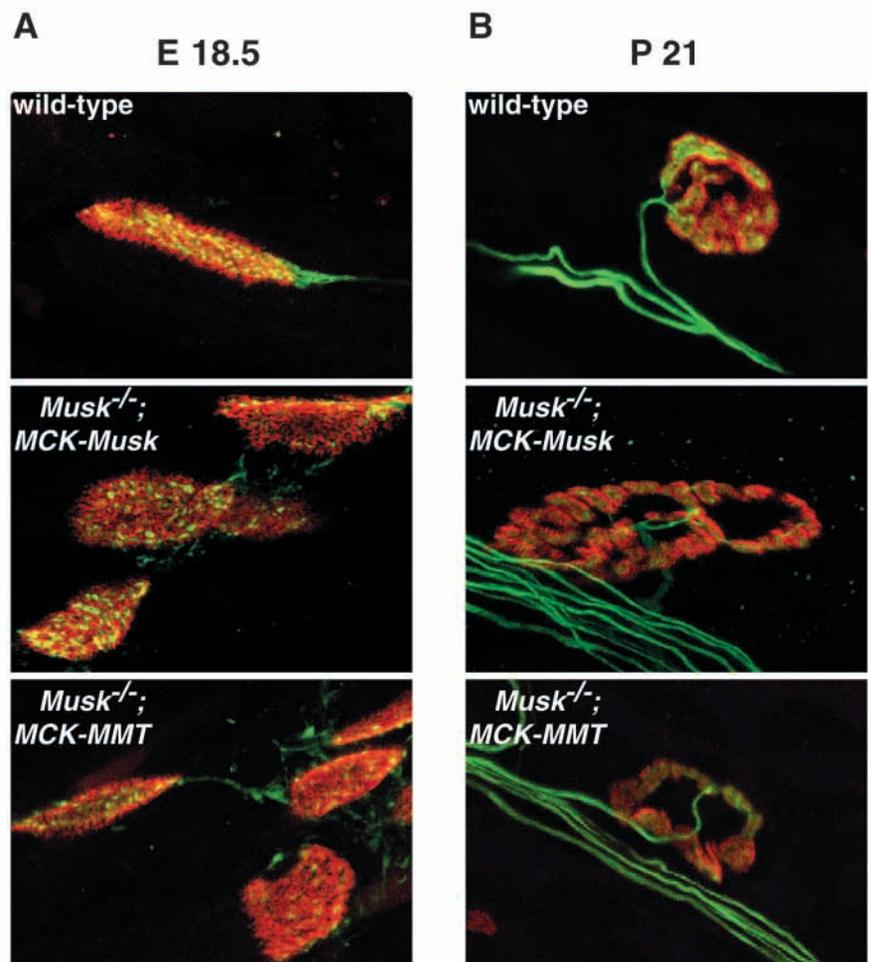

Fig. 2. Synapse formation is rescued in $Musk$ mutant mice expressing $MCK\text{-}MMT$ or $MCK\text{-}Musk$. (A) Motor axons terminate at AChR clusters in muscle from E18.5 $Musk$ mutant embryos carrying $MCK\text{-}MMT$ or $MCK\text{-}Musk$. In E18.5 $Musk$ mutant embryos, motor axons fail to stop or differentiate, and AChR clusters fail to form. (B) Synapses are singly innervated in muscle from 3-week-old $Musk^{-/-}$; $MCK\text{-}MMT$ mice, similar to wild-type and $Musk^{-/-}$; $MCK\text{-}Musk$ mice. Wholemounts of diaphragm muscles were stained with antibodies to neurofilament (green) and synaptophysin (green) to label axons and presynaptic nerve terminals, respectively, and with Texas-Red α-Bungarotoxin (red) to label AChRs. Scale bar:15 μm in A; 20 μm in B.
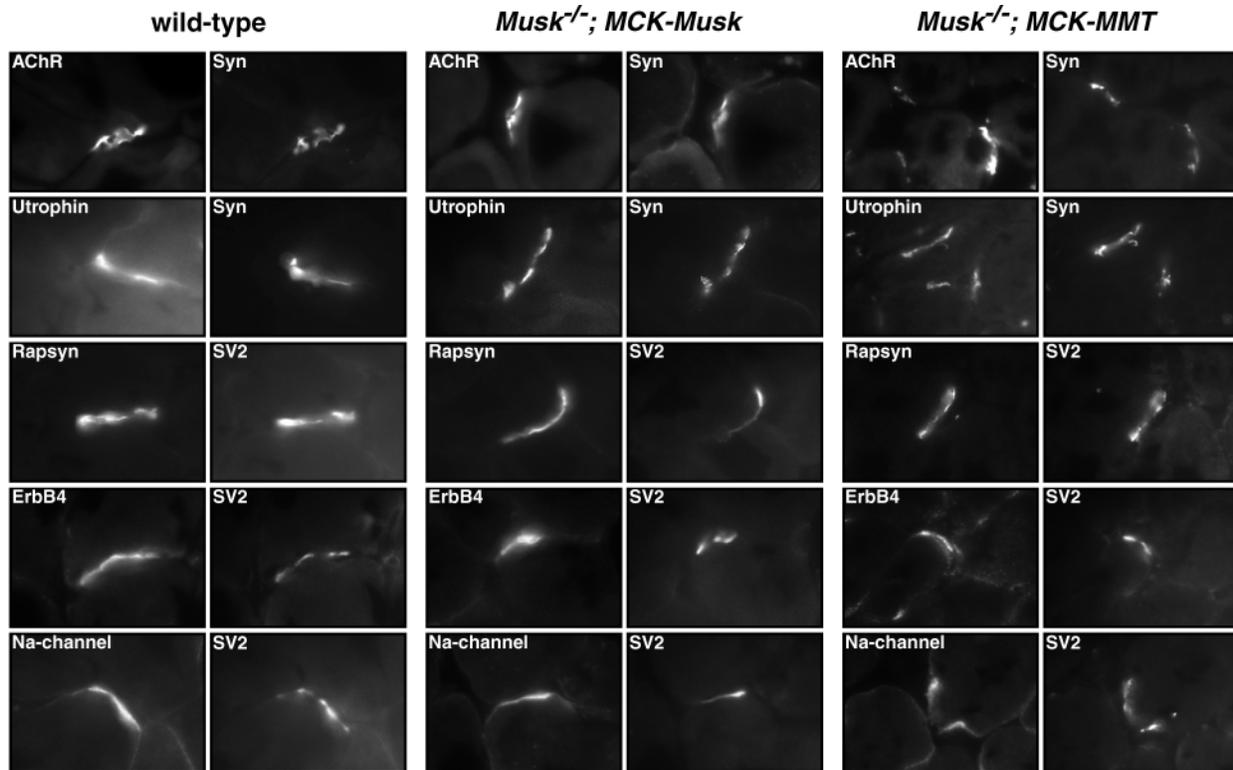
Fig. 3. Postsynaptic proteins are clustered at synaptic sites in *Musk* mutant mice expressing *MCK-MMT* (right) Synaptic proteins, including rapsyn, ErbB4, Na$^+$ channels and utrophin, co-localize with nerve terminals in wild-type, *Musk$^{-/-}$; MCK-Musk* and *Musk$^{-/-}$; MCK-MMT* mice. Sections from muscles of 3-week-old mice were stained with antibodies to postsynaptic proteins and synaptophysin (Syn) or SV2 to label presynaptic nerve terminals.

AChR α and δ subunit mRNAs are each enriched in the central region of intercostal muscle from *Musk$^{-/-}$*; *MCK-MMT* newborn mice, demonstrating that the Musk juxtamembrane region has a key role in activating a pathway leading to synapse-specific gene expression.

Musk and MMT proteins are concentrated at synaptic sites despite uniform *Musk* and *MMT* RNA expression

In wild-type mice, *Musk* RNA, like AChR mRNA, is expressed preferentially at synaptic sites (Valenzuela et al., 1995). Because the *MCK* gene is expressed in nuclei throughout the myofiber (Tang et al., 1994), the *MCK* regulatory region, as expected, confers *Musk* and *MMT* mRNA expression uniformly throughout the muscle (Fig. 6). We examined whether Musk protein, encoded by the transgene, is expressed uniformly in the muscle or concentrated at synaptic sites in *Musk$^{-/-}$; MCK-Musk* mice. We stained sections of muscle from *Musk$^{-/-}$; MCK-Musk* mice with antibodies to Musk and found that Musk is clustered at synaptic sites (Fig. 6). Likewise, we found that MMT is clustered at synaptic sites in *Musk$^{-/-}$; MCK-MMT* mice (Fig. 6). Thus, Musk protein can be clustered at synaptic sites even if *Musk* mRNA is expressed uniformly in muscle.

Motor axons extend until Musk is expressed, yet postsynaptic differentiation is limited to a discrete site on the muscle fiber

Motor axons enter developing skeletal muscle at E12.5 and form a main intramuscular nerve in the middle of the muscle (Sanes and Lichtman, 1999). In wild-type mice, motor axons branch and terminate adjacent to the main intramuscular nerve, resulting in a narrow, distinct endplate zone in the middle of the muscle, marked by presynaptic nerve terminals and a high concentration of AChRs (Fig. 7). In *Musk* mutant mice, motor axons branch from the main intramuscular nerve, but these axons fail to terminate and instead wander across the muscle surface without forming specialized nerve terminals (DeChiara et al., 1996).

Based on the expression of transgenes containing the MCK enhancer and promoter, the endogenous *MCK* gene is activated in skeletal muscle at ~E15.5 (S. Hauschka, personal communication), 1 day after motor axons first enter the muscle. Expression of the *MCK* gene increases modestly (~20-fold) between E13.5 and birth, and dramatically after birth (>300-fold between birth and P21) (S. Hauschka, personal communication). We therefore reasoned that axon growth would be exuberant in *Musk$^{-/-}$; MCK-Musk* mice, as in *Musk* mutant mice, prior to the onset of Musk expression. Fig. 7 shows that motor axon growth is similarly exuberant in *Musk$^{-/-}$*, *Musk$^{-/-}$; MCK-Musk* and *Musk$^{-/-}$; MCK-MMT* mice at E14.5 and E16.5. In addition, postsynaptic clustering of AChRs is absent at E14.5 and only weakly detectable at E16.5 in *Musk$^{-/-}$; MCK-Musk* and *Musk$^{-/-}$; MCK-MMT* mice. Between E16.5 and E18.5 in *Musk$^{-/-}$; MCK-Musk* mice, the rate of axon growth reverts to normal and postsynaptic differentiation commences (Fig. 7). In *Musk$^{-/-}$*;
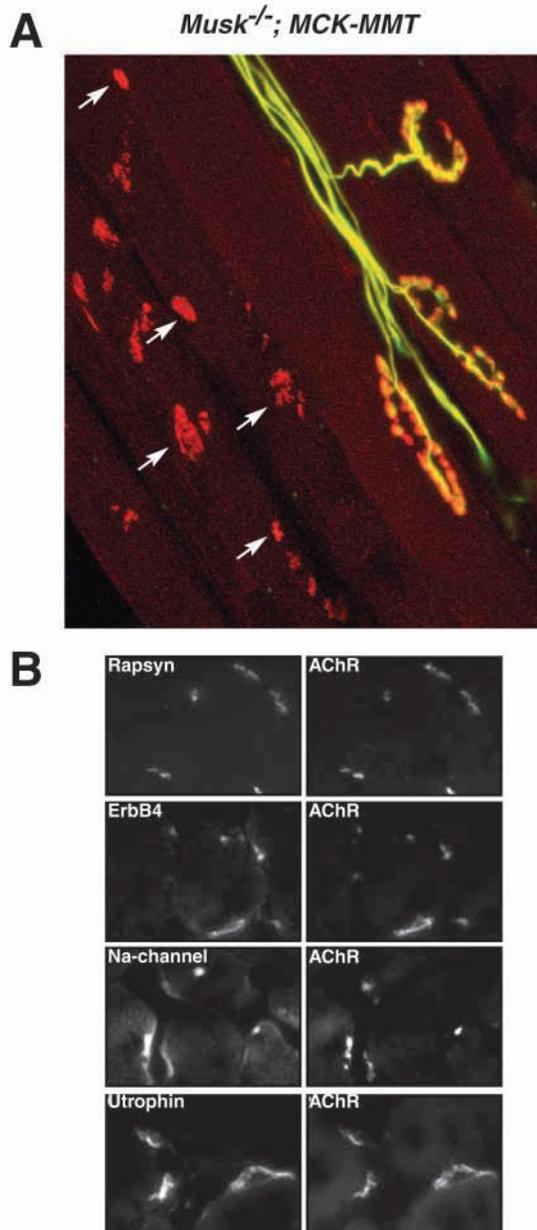
Fig. 4. *MCK-MMT* induces ectopic, postsynaptic-like specializations. (A) In *Musk⁻/⁻; MCK-MMT* mice, AChRs are clustered not only at synaptic sites but also at non-synaptic sites (arrows). Wholemounts of diaphragm muscles from 3-week-old mice were stained with antibodies to neurofilament (green) and synaptophysin (green) to label axons and presynaptic nerve terminals, and with Texas-Red α-Bungarotoxin (red) to label AChRs. (B) Nonsynaptic AChR clusters co-localize with other 'synaptic' proteins, including rapsyn, ErbB4, Na⁺ channels and utrophin. Sections from muscles of 3-week-old mice were stained with antibodies to postsynaptic proteins and with Texas Red α-Bungarotoxin to label AChRs. In muscle from wild-type and *Musk⁻/⁻; MCK-Musk* mice, nerve terminal staining reliably coincides with AChR clusters. In *Musk⁻/⁻; MCK-MMT* mice, nerve terminal staining coincides with only a subset of AChR clusters.

Fig. 5. AChR subunit genes are transcribed selectively in subsynaptic nuclei of *Musk* mutant mice expressing *MCK-Musk* and *MCK-MMT*. AChR α and δ mRNAs are expressed preferentially in the central, synaptic region of muscle (arrows) in wild-type mice and uniformly in skeletal muscle of *Musk⁻/⁻* mice. In *Musk⁻/⁻; MCK-Musk* and *Musk⁻/⁻; MCK-MMT* mice, AChR α and δ mRNAs are expressed preferentially in the central region of the muscle. The pattern of AChR transcription was assessed by in situ hybridization of intercostal muscle from E18.5 embryos.

MCK-MMT mice, the rate of axon outgrowth also reverts between E16.5 and E18.5, but incompletely, like AChR clustering. Owing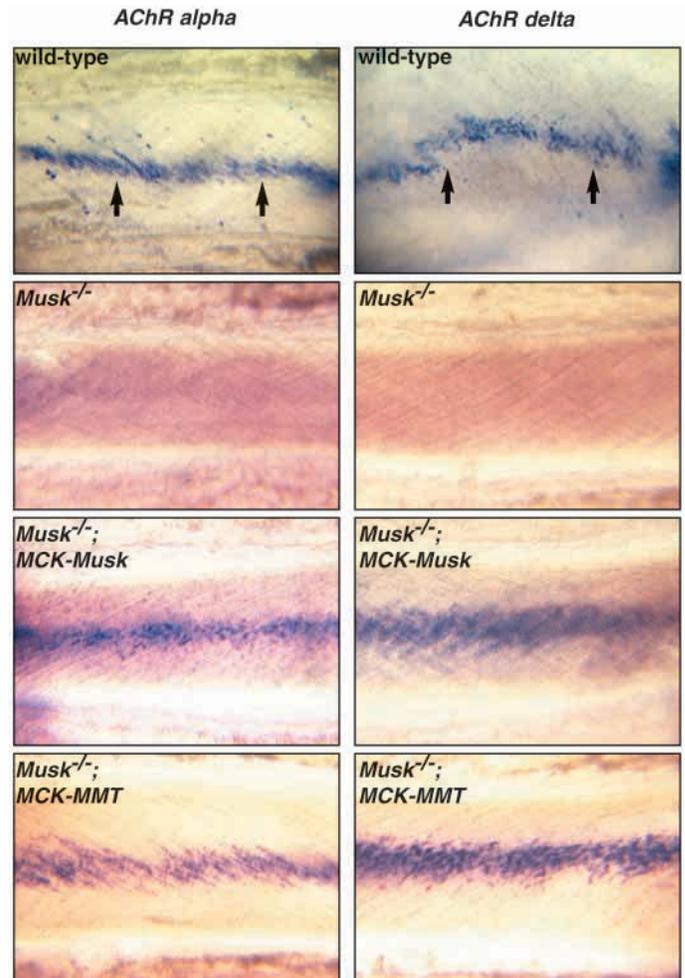 to the late expression of the *MCK* gene, motor axons extend well beyond their normal termination zone prior to forming neuromuscular synapses, resulting in a substantially wider end-plate zone, which persists in rescued adult mice (Fig. 8).

Because motor axons in *Musk⁻/⁻*; *MCK-Musk* mice grow over a substantial region of muscle prior to E16.5, we reasoned that postsynaptic differentiation, initiated by neurally deposited agrin, might occur over an unusually large region of each muscle fiber once *Musk* expression began. We found, however, that the size of AChR clusters was normal (102±4%, *n*=3) in muscle from *Musk⁻/⁻*; *MCK-Musk* newborn mice (Fig. 2). These results raise the possibility that agrin is preferentially available, or active, at or near the growth cone of the motor axon, and that the resulting bias in Musk activation consolidates Musk and AChR clustering to a discrete patch on the muscle fiber surface (see Discussion).

Terminal arbors are immature in *Musk*^{−/−}; *MCK-MMT* mice

Neuromuscular synapses undergo several structural and functional transitions during the first few weeks after birth, as the number of presynaptic inputs at individual synapses is reduced to one, and the single, remaining nerve terminal arbor becomes more complex (Sanes and Lichtman, 1999). To determine whether synaptic sites become singly innervated and nerve terminal arbors become more complex in *Musk* mutant mice expressing MMT, we stained wholemounts of diaphragm

muscle from 3-week-old mice with antibodies to neurofilament/synaptophysin and with TR-α-BGT. In *Musk*^{−/−}; *MCK-Musk* and *Musk*^{−/−}; *MCK-MMT* mice, as in wild-type mice, synaptic sites are singly innervated (Fig. 2B, Fig. 9). Thus, synapse elimination appears normal. In *Musk*^{−/−}; *MCK-Musk* mice, motor axon terminals arborize and invariably form a complex, branched endplate (Fig. 2B, Fig. 9). In muscle from *Musk*^{−/−}; *MCK-MMT* mice, however, terminal branching is less extensive, resulting in less complex terminal arbors (Fig. 2B, Fig. 9). Some (~10%) terminal arbors are remarkably simplified, as terminal branches are fragmented into individual boutons (Fig. 9). These results indicate that MMT can restore presynaptic differentiation, although the extent of terminal arborization is incomplete. Because terminal arbor differentiation and AChR clustering are restored to a similar degree in *Musk*^{−/−}; *MCK-MMT* mice, Musk activity in the postsynaptic cell appears to be limiting for presynaptic differentiation.

DISCUSSION

Agrin stimulates the phosphorylation of five tyrosine residues in the Musk kinase domain and one tyrosine residue, Y553, in the juxtamembrane region of Musk (Watty et al., 2000). Previously, we generated chimeric proteins, composed of the extracellular and transmembrane domains of Musk, and the intracellular domain of TrkA, and we found that inclusion of thirteen amino acids from the juxtamembrane region of Musk, including Y553, is sufficient, even in the context of the TrkA kinase domain, to activate a signaling pathway leading to the clustering and tyrosine phosphorylation of AChRs in cultured myotubes (Herbst and Burden, 2000). We show that muscle-specific expression of this chimeric protein rescues *Musk* mutant mice, as mice that lack endogenous Musk but express the chimeric protein move, breathe and survive after birth. Expression of the chimeric protein restores neuromuscular synapse formation: nerve terminal arbors develop, postsynaptic
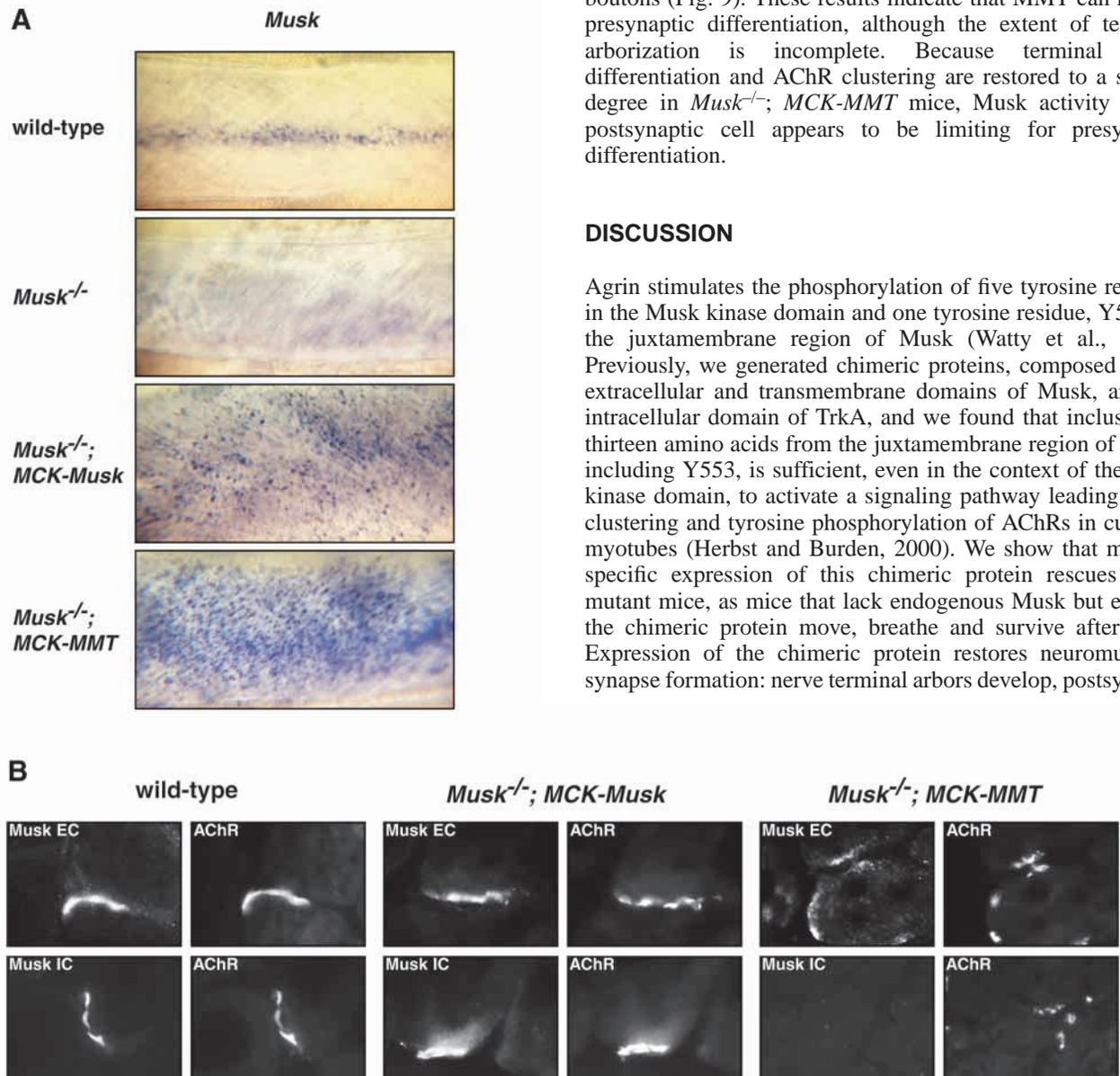

Fig. 6. Musk and MMT protein are concentrated at synaptic sites, despite uniform mRNA expression in *Musk*^{−/−}; *MCK-Musk* and *Musk*^{−/−}; *MCK-MMT* mice. (A) *Musk* mRNA is expressed preferentially in the central, synaptic region of muscle in wild-type mice and at uniform, low levels in muscle from *Musk* mutant embryos. In *Musk*^{−/−}; *MCK-Musk* and *Musk*^{−/−}; *MCK-MMT* embryos, *Musk* and *MMT* mRNA, respectively, are expressed uniformly in muscle. The pattern of *Musk* or *MMT* transcription was assessed by in situ hybridization of intercostal muscle from E18.5 embryos. (B) Sections from muscle of 3-week-old mice were stained with antibodies to Musk (EC, antibodies, 83033, to an extracellular epitope; IC, antibodies, 24908, to an intracellular epitope) and with Texas Red α-Bungarotoxin to label AChRs. Musk and MMT proteins are concentrated at postsynaptic sites despite uniform expression of *Musk* and *MMT* RNAs throughout the muscle. IC does not recognize MMT, as MMT lacks the epitope from the C terminus of Musk.
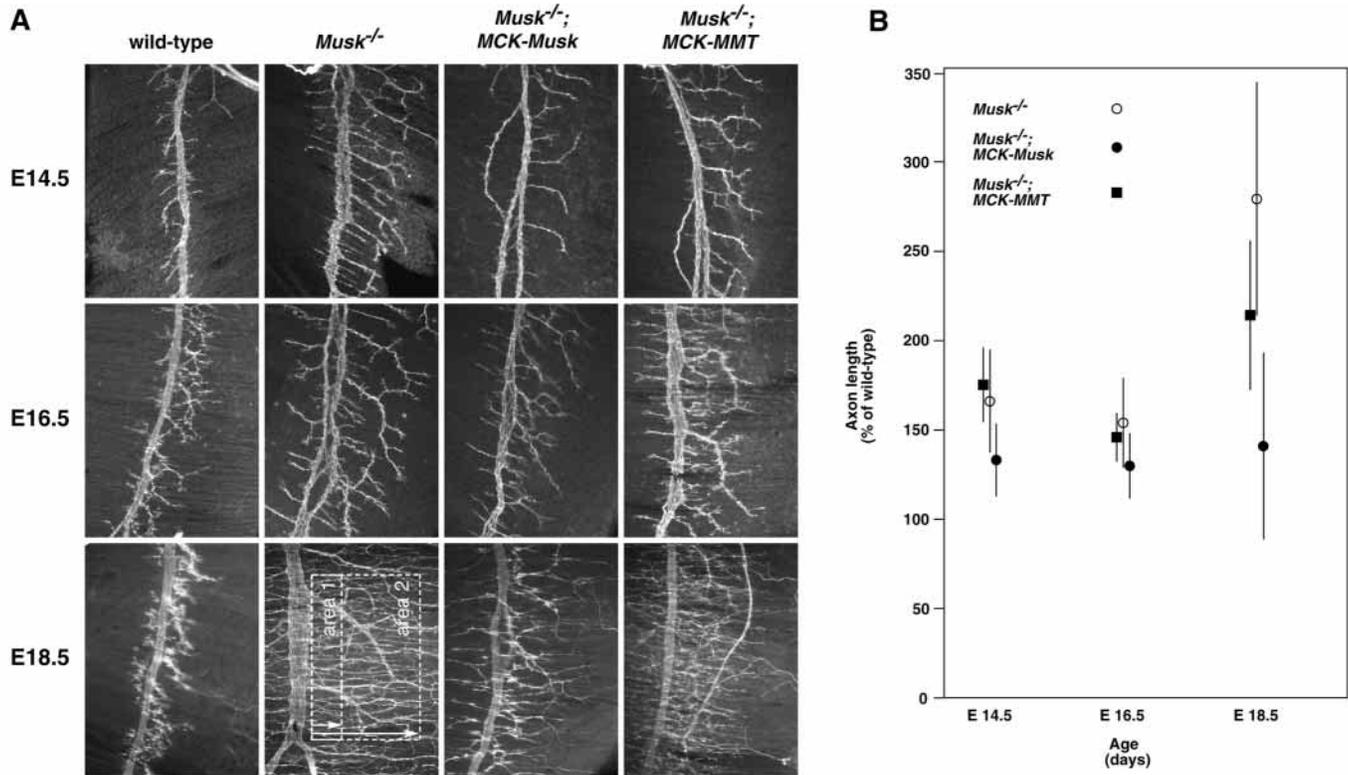
Fig. 7. Timing of *MCK* expression correlates with cessation of axon growth. In E14.5 and E16.5 *Musk⁻/⁻; MCK-Musk* and *MCK⁻/⁻; MCK-MMT* embryos, motor axon growth proceeds well beyond the normal termination zone, similar to *Musk* mutant embryos. Motor axon growth ceases and nerve terminals differentiate only after *Musk*, or *MMT*, is expressed from the *MCK* regulatory region, between E16.5 and E18.5. Wholemounts of diaphragm muscles from E14.5, E16.5 and E18.5 embryos were stained with antibodies to neurofilament (NF) and synaptophysin (Syn) to label axons and presynaptic nerve terminals, respectively. (B) Quantitation of axon growth shows that motor axons stop in *Musk⁻/⁻; MCK-Musk* embryos at ~E16.5, when *MCK* expression begins. Likewise, in *MCK⁻/⁻; MCK-MMT* embryos, exuberant motor axon growth reverts at ~E16.5, but this reversion is partial as motor axon growth at E18.5 is intermediate between that in wild-type and *Musk* mutant embryos.

proteins, including AChRs, cluster at synaptic sites, and AChR genes are transcribed selectively in synaptic nuclei. Kinase activity per se is insufficient to activate synaptic differentiation, as chimeric RTKs lacking the Musk juxtamembrane region fail to activate synaptic differentiation in cultured muscle cells and at ectopic sites in muscle in vivo (Jones et al., 1999; Herbst and Burden, 2000). Thus, the Musk juxtamembrane region has a key role in activating the multiple pathways leading to presynaptic and postsynaptic differentiation in vivo.

The precise role for Musk in synapse-specific expression is poorly understood. Analysis of rapsyn mutant mice indicates that Musk may activate a signaling pathway that directly stimulates synapse-specific transcription (Gautam et al., 1995). Expression of ectopic agrin or activated Musk in adult myofibers, however, stimulates AChR transcription in an ErbB-dependent manner, suggesting that synapse-specific expression may require the Musk-dependent recruitment of a Nrg-1/ErbB signaling complex to synaptic sites (Jones et al., 1999; Meier et al., 1997; Moore et al., 2001; Rimer et al., 1998). Our experiments indicate that the Musk juxtamembrane region contains sequences that are crucial for synapse-specific transcription, but they do not shed light on whether these sequences act in a manner that is independent or dependent on Nrg-1/ErbB signaling. Thus, the Musk juxtamembrane region could recruit a signaling complex that directly regulates

transcription in synaptic nuclei, or the Musk juxtamembrane region could regulate synapse-specific transcription indirectly by regulating the distribution of a Nrg-1/ErbB signaling complex, analogous to the action of Musk on AChR protein. In either case, our experiments indicate that the Musk juxtamembrane region, rather than other potential phosphotyrosine docking sites in the Musk kinase domain, has a crucial role in synapse-specific transcription.

In wild-type mice, *Musk* mRNA and protein are concentrated at neuromuscular synapses (Valenzuela et al., 1995). In *Musk* mutant mice carrying *MCK-Musk*, *Musk* mRNA is expressed throughout the muscle, yet Musk protein is concentrated at synaptic sites. These experiments demonstrate that accumulation of Musk protein at synaptic sites is not dependent upon synaptic localization of *Musk* mRNA. Thus, it may be more important that muscle fibers express an adequate level of mRNAs encoding synaptic proteins, such as Musk and AChR, rather than restricting these mRNAs to synaptic sites. Moreover, in *Musk* mutant mice carrying *MCK-MMT*, *MMT* mRNA is likewise expressed throughout the muscle, yet the chimeric protein is concentrated at synaptic sites. These results suggest that activation of the chimeric protein by agrin is sufficient to recruit additional chimeric protein from non-synaptic regions, via a positive feedback mechanism (Jones et al., 1999). Our findings, both
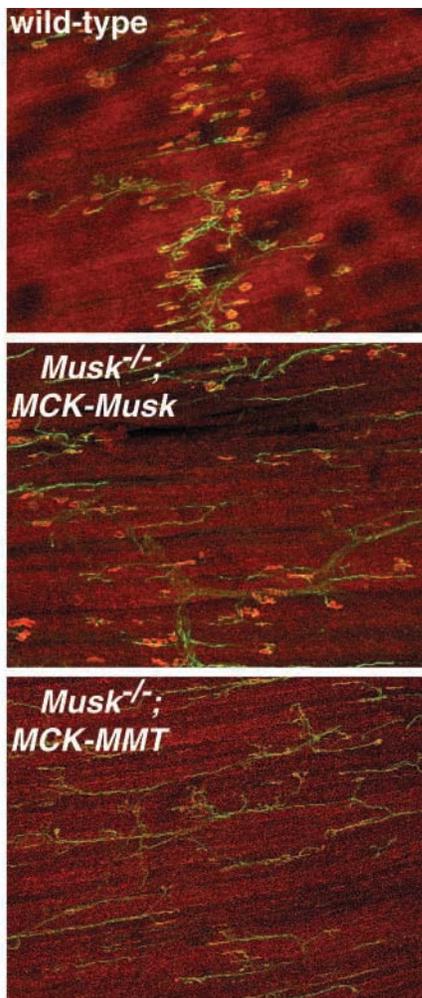
Fig. 8. The synaptic zone remains broader in *Musk⁻/⁻; MCK-Musk* and *Musk⁻/⁻; MCK-MMT* adult mice. As a consequence of delayed expression from the *MCK* gene, the axon termination zone is wider in *MCK⁻/⁻; MCK-Musk* and *MCK⁻/⁻; MCK-MMT* embryos than in wild-type embryos, and this broader termination zone is retained in adult mice. Wholemounts of diaphragm muscles from 3-week-old mice were stained with antibodies to neurofilament (NF) and synaptophysin (Syn) to label axons and presynaptic nerve terminals (green), and with Texas-Red α-Bungarotoxin (TR-α-BGT) to label AChRs (red).

Fig. 9. Synaptic sites are singly innervated in *Musk* mutant mice expressing *MCK-MMT* but nerve terminal arbors are simplified. Synapses are singly innervated in muscle from 3-week-old *Musk⁻/⁻; MCK-MMT* mice, similar to wild-type and *Musk⁻/⁻; MCK-Musk* mice. Nerve terminals are similarly arborized in wild-type and *Musk⁻/⁻; MCK-Musk* mice (A-C), but in *Musk⁻/⁻; MCK-MMT* mice, nerve terminal differentiation is incomplete, ranging from terminals with less complex arbors (D) to nerve terminals with strikingly simplified arbors and bouton-like endings (E,F). Wholemounts of diaphragm muscles from three 3-week-old mice were stained with antibodies to neurofilament (NF) and synaptophysin (Syn) to label axons and presynaptic nerve terminals, respectively, and with Texas-Red α-Bungarotoxin (TR-α–BGT) to label AChRs. Scale bar: 20 μm.

from in vitro and in vivo experiments, therefore, underscore the crucial role of the Musk juxtamembrane domain in clustering Musk as well as AChRs. The mechanisms by which activated Musk recruits Musk are not understood, but this positive feedback loop may be important for achieving an adequate level of Musk expression at the synapse, sufficient to cluster more than ten million AChR molecules per synapse. Nonetheless, it seems likely that this positive feedback mechanism is restrained, as Musk activation and clustering would otherwise proceed beyond the synaptic site.

We showed previously that the MMT chimera is less responsive than Musk to agrin, as agrin stimulates half the number of AChR clusters in MMT-expressing myotubes th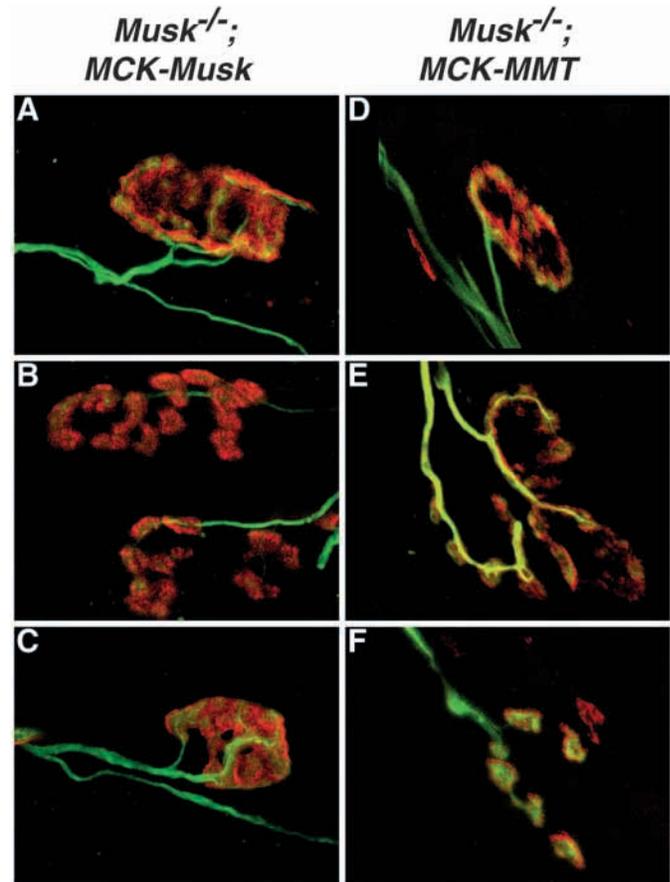an in Musk-expressing myotubes (Herbst and Burden, 2000). We found that the density of synaptic AChRs is lower in *Musk* mutant mice rescued with MMT than in *Musk* mutant mice rescued with Musk. The lower density of synaptic AChRs could be due to the reduced responsiveness of MMT to agrin, poorer (approx. half) expression of the MMT transgene, or both. Because MMT is less responsive to agrin in vitro, we favor the idea that sequences in the intracellular domain of Musk, not present in the MMT chimera, contribute to clustering of AChRs at synapses as well. Consistent with this idea, Y576, in the N-terminal lobe of the kinase domain, is phosphorylated in activated Musk and contributes to AChR clustering in cultured muscle cells (Herbst and Burden, 2000). Alternatively, maximal AChR clustering may depend upon sequences in Musk, not represented in the MMT chimera, that bind proteins independently of tyrosine phosphorylation (Strochlic et al., 2001).

We showed previously that MMT-expressing myotubes have more agrin-independent AChR clusters than Musk-expressing myotubes (Herbst and Burden, 2000). Consistent with these results, we found ectopic AChR clusters in innervated myofibers from *Musk* mutant mice rescued with MMT but not in *Musk* mutant mice rescued with Musk. These data support the idea that sequences in Musk restrain Musk activation in vivo, and that the absence of this auto-inhibitory sequence in the MMT chimera results in adventitious Musk activity (Till et al., 2002).

During the first few weeks after birth, the structure and function of the neuromuscular synapse is modified (Sanes and Lichtman, 1999). At birth, multiple motor axons terminate at a single, elliptical synaptic site on each myofiber. During the next few weeks, all but one of these motor axons is withdrawn, leading to innervation of the single synaptic site by a single motor axon. In addition, the shape of the synaptic site becomes more complex, as the presynaptic terminal grows and branches, and the shape of synaptic AChR clusters, ovoid at birth, becomes correspondingly complex. Although the elimination of polyneuronal innervation appears to occur normally in *Musk* mutant mice expressing *MCK-MMT*, presynaptic terminal arbors are often less complex and lack extensive terminal branching. Indeed, some terminal arbors are so simplified that the synaptic site is composed of only a few boutons. These aberrations in terminal branching could arise from a failure to branch adequately when synapses first form or from remodeling and simplification, rather than growth and elaboration of branches later in development (Balice-Gordon and Lichtman, 1990). In either case, presynaptic differentiation, like postsynaptic differentiation, is incomplete in *Musk* mutant mice expressing *MCK-MMT*, and these results reinforce the idea that sequences other than the critical juxtamembrane region of Musk, though not essential for synapse formation, contribute to postsynaptic differentiation and presynaptic terminal arborization. Moreover, although prior studies of *rapsyn* and *Musk* mutant mice demonstrated that presynaptic differentiation is linked to postsynaptic differentiation (DeChiara et al., 1996; Gautam et al., 1995), the experiments described demonstrate that the extent of presynaptic and postsynaptic differentiation are matched, indicating that Musk activity in the postsynaptic cell is limiting for presynaptic differentiation. Despite the simplified structure of synapses in *Musk*^{-/-}; *MCK-MMT* mice, these mice are viable, fertile and behave normally in simple behavioral paradigms (see Materials and Methods).

Musk mutant mice carrying a *MCK-Musk* transgene first express Musk when motor axons have already extended well beyond their normal termination zone. Thus, it seemed possible that agrin, which is deposited by motor axons growing exuberantly along the muscle, might initiate postsynaptic differentiation over an unusually extensive area of the muscle once Musk expression begins. The shape and size of AChR clusters, however, are similar in wild-type mice and in *Musk* mutant mice that express *MCK-Musk*. The mechanisms that regulate release and retention of agrin from motor axons are poorly understood (Cohen et al., 1994; Ma et al., 2000), but our results are consistent with the idea that agrin accumulates, or is more active, at or near the growth cone and that an ensuing bias in Musk activation leads to focal clustering of Musk and AChRs.

The timing of *MCK-Musk* expression correlates well with the cessation of motor axon growth and the onset of nerve terminal differentiation in *Musk*^{-/-}; *MCK-Musk* mice. Prior to transgene expression, motor axon growth proceeds well beyond the normal synaptic zone in the muscle. Motor axon growth halts and the differentiation of nerve terminals begins in *Musk*^{-/-}; *MCK-Musk* and *Musk*^{-/-}; *MCK-MMT* mice, only after Musk, or MMT, is expressed from the *MCK* regulatory region. These results confirm prior studies of *Musk* and agrin mutant mice and provide direct evidence that Musk regulates the organization, or synthesis of a stop signal(s) for axon growth and nerve terminal differentiation.

The C terminus of Musk contains a binding-site for PDZ domain-containing proteins. Clustering of AChRs, at least in cultured muscle cells, however, is not dependent upon this sequence, as agrin stimulates AChR clustering in myotubes expressing a C-terminal Musk mutant (Zhou et al., 1999). As the *MCK-Musk* transgene, which is studied here, is a fusion between *Musk* and *GFP*, this gene fusion encodes a protein that lacks a C-terminal binding site for PDZ domains. Because this transgene fully rescues the presynaptic and postsynaptic defects of *Musk* mutant mice, our experiments indicate that recruitment of PDZ domain-containing proteins to the C-terminus of Musk is not required for Musk to stimulate presynaptic and postsynaptic differentiation in vivo.

In chick and *Xenopus*, *Musk* expression is not restricted to skeletal muscle. In chick embryos, *Musk* is expressed transiently in the brain and liver (Ip et al., 2000), and in *Xenopus* embryos, Musk is expressed widely in the CNS (Fu et al., 1999). These findings raised the possibility that low and/or transient Musk expression in motoneurons of mice may have escaped attention and that a loss of motorneuron-derived Musk could be responsible for the presynaptic deficits in *Musk* mutant mice. We find that muscle-specific expression of Musk is sufficient to restore presynaptic as well as postsynaptic differentiation in *Musk* mutant mice. Thus, these data strongly support the idea that activation of Musk in skeletal muscle is required to initiate a signaling pathway that leads to production and/or clustering of a retrograde signal for presynaptic differentiation.

The steps that follow Musk activation and that lead to neuromuscular synapse formation are poorly understood (Mittaud et al., 2001; Mohamed et al., 2001; Smith et al., 2001; Weston et al., 2000). Our results indicate that phosphorylation of the Musk juxtamembrane region initiates a signaling pathway that regulates nerve terminal differentiation, synapse-specific transcription and clustering of postsynaptic proteins. Thus, the Musk juxtamembrane region, including a single phosphotyrosine docking site, controls multiple pathways leading to presynaptic and postsynaptic differentiation in vivo.

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