Wnt Signals Organize Synaptic Prepattern and Axon Guidance through the Zebrafish *unplugged*/MuSK Receptor

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SUMMARY

Early during neuromuscular development, acetylcholine receptors (AChRs) accumulate at the center of muscle fibers, precisely where motor growth cones navigate and synapses eventually form. Here, we show that Wnt11r binds to the zebrafish *unplugged*/MuSK ectodomain to organize this central muscle zone. In the absence of such a zone, prepatterned AChRs fail to aggregate and, as visualized by live-cell imaging, growth cones stray from their central path. Using inducible *unplugged*/MuSK transgenes, we show that organization of the central muscle zone is dispensable for the formation of neural synapses, but essential for AChR prepattern and motor growth cone guidance. Finally, we show that blocking noncanonical *dishevelled* signaling in muscle fibers disrupts AChR prepatterning and growth cone guidance. We propose that Wnt ligands activate *unplugged*/MuSK signaling in muscle fibers to restrict growth cone guidance and AChR prepatterns to the muscle center through a mechanism reminiscent of the planar cell polarity pathway.

INTRODUCTION

Formation of functional neuromuscular synapses requires the interplay between presynaptic nerves and postsynaptic muscle components (Burden, 2002; Sanes and Lichtman, 2001). In vertebrates, a hallmark of neuromuscular synapses is the accumulation of acetylcholine receptors (AChRs) in a narrow, central region of muscle fibers, in apposition to nerve terminals. Development of neuromuscular synapses requires nerve-derived agrin to counteract the acetylcholine-mediated dispersal of AChR clusters (Lin et al., 2005; Misgeld et al., 2005). This leads to the removal of aneural AChR clusters and the stabilization of nerve-terminal-associated AChR clusters, i.e., nascent synapses. Postsynaptic differentiation also requires the muscle-specific receptor tyrosine kinase MuSK, a component of the MuSK/Lrp4 agrin receptor, to promote AChR clustering and activate AChR gene expression (DeChiara et al., 1996; Glass et al., 1996; Kim et al., 2008; Zhang et al., 2008).

Even before motor axons contact muscle fibers, AChR clusters are localized to the central region of muscle, independent of nerve contact or nerve-derived agrin (Lin et al., 2001; Yang et al., 2001). This AChR prepattern requires MuSK function, and recent studies suggest that ectopic MuSK expression is sufficient to establish AChR prepatterning (Kim and Burden, 2008). Upon contact with motor axons, pre-existing AChR clusters are incorporated into prospective neuromuscular synapses (Flanagan-Steet et al., 2005; Panzer et al., 2006). Thus, formation of neuromuscular synapses can be divided into two phases. An early phase when AChRs first cluster in the center of muscle fibers, precisely where motor growth cones will navigate, and a later phase, when growth cones have made contact with muscle fibers and neural AChR clusters become incorporated into functional neuromuscular synapses (Lin et al., 2001).

Over the past decades, many of the molecular players and mechanisms involved in the later phase of neuromuscular synapse development have been discovered, while the molecules and mechanisms underlying the early phase are not well understood (Burden, 2002; Burden et al., 2003; Kummer et al., 2006; Sanes and Lichtman, 2001). For example, what is the role of nerve-independent postsynaptic differentiation, i.e., AChR prepattern during normal synaptogenesis? Similarly, what initiates AChR prepattern and determines its central muscle location? Here, we provide compelling evidence that in zebrafish embryos *wnt11r* is required to confine navigating growth cones to the center of muscle fibers and to initiate AChR prepattern. We show that *wnt11r* and *unplugged* interact genetically, that Wnt11r binds Unplugged/MuSK through its *frizzled*-like cysteine-rich domain (CRD) in vitro, that in the embryo *wnt11r* binds exclusively to the *unplugged* receptor expression domain in an *unplugged*/MuSK-dependent manner, and that noncanonical *dishevelled* signaling in muscle fibers is required for *Unplugged*/MuSK function. Together, our data provide strong evidence that Wnt ligands activate *unplugged*/MuSK signaling in muscle fibers to organize a central muscle zone and thereby spatially restrict growth cone guidance and AChR accumulation through a mechanism reminiscent of the planar cell polarity pathway.

RESULTS

unplugged SV1 Is Required for AChR Prepattern

In the zebrafish embryo, the first AChR prepattern forms on adaxial muscle cells, initially located on the medial surface of

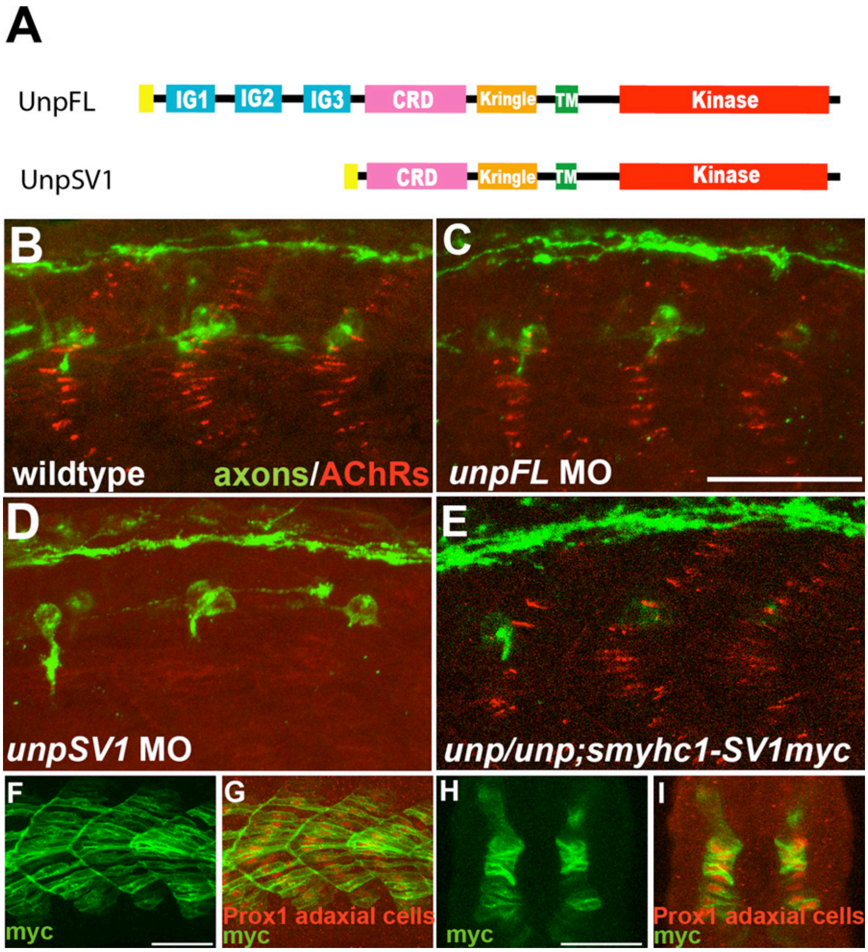

Figure 1. UnpSV1 Controls AChR Prepatterning

(A) Domain structure of the Unplugged protein isoforms.

(B–E) Lateral views of caudal segments in 17 hpf embryos stained for motor axons (green, znp-1/SV2) and AChRs (red, α-BTX). (B) In wild-type embryos, AChRs are prepatterned in a central band along the dorsal and ventral myotome before the first growth cones approach. (C) UnpFL MO injection does not affect AChR prepattern. (D) UnpSV1 MO injection causes complete absence of AChR prepattern. (E) UnpSV1 expression in adaxial cells restores AChR prepattern in unplugged mutant embryos.

(F and G) Lateral views and cross-sectional views (H and I) of 17 hpf Tg(smyhc1:UnpSV1myc) embryos stained with anti-myc (green) and anti-Prox1 (red), which labels the nuclei of adaxial cells. Scale bars, 50 μm.

somites (Flanagan-Steet et al., 2005; Panzer et al., 2005). As motor growth cones enter the muscle, migratory adaxial cells delaminate from the medial surface, and lateral fast muscle fibers invade the space on the medial somite surface (Figures S1A and S1B available online and Cortes et al., 2003). Motor growth cones then contact medial fast muscle fibers and form neural en passant synapses at sites previously marked by prepatterned AChRs (Flanagan-Steet et al., 2005; Panzer et al., 2005). A group of two to five nonmigratory adaxial cells, termed muscle pioneers, remain on the medial myotome surface, and upon contact with motor growth cones, form the first neuromuscular junctions (NMJs) (Figure S1B and Flanagan-Steet et al., 2005; Liu and Westerfield, 1992). Here, we focus on the formation of the adaxial cell AChR prepattern.

We have previously shown that unplugged null mutants lack all AChR prepatterning and en passant neuromuscular junctions and that they display specific axonal guidance defects (Lefebvre et al., 2007; Zhang et al., 2004). The zebrafish unplugged locus encodes two MuSK isoforms: the unplugged full-length (FL) isoform, which is essential for the formation of neuromuscular junctions, and the unplugged splice variant 1 (SV1) isoform, which is essential for axonal guidance, independent of rapsyn (Figure 1A and Zhang et al., 2004). To examine the role of both isoforms during AChR prepatterning in adaxial fibers, we first analyzed the expression patterns of unplugged FL and SV1 using isoform-specific probes. Before and during the time of AChR prepatterning, unplugged FL is weakly expressed while unplugged SV1 is strongly expressed in adaxial cells (Figures S1C–S1F). Thus, unplugged SV1 expression is consistent with a role in adaxial AChR prepattern.

Besides their expression patterns, the two unplugged isoforms also differ in their ectodomain composition. While the FL isoform contains three immunoglobulin-like (Ig) domains in addition to the CRD and the kringle domain, the SV1 ectodomain lacks the Ig domains and only consists of a unique signal sequence followed by the CRD and the kringle domain (Figure 1 and Zhang et al., 2004). To determine which of the two unplugged/MuSK isoforms is critical to initiate adaxial AChR prepatterning in vivo, we used a set of morpholinos previously shown to affect one but not the other isoform (Lefebvre et al., 2007; Zhang et al., 2004). Morpholino-mediated knockdown of unplugged FL and SV1 revealed that unplugged SV1 but not FL is essential for prepatterning, consistent with their differential expression patterns (Figures 1B–1D). To confirm that the unplugged SV1 receptor is indeed responsible for AChR prepatterning, we generated transgenic lines in which myc-tagged unplugged SV1 is expressed under the control of a slow myosin heavy chain (smyhc1) promoter specific for adaxial cells (Figures 1F–1I and Elworthy et al., 2008). When crossed into the unplugged mutant background, the presence of Tg(smyhc1:unpluggedSV1-myc) in unplugged$^{(br307/br307)}$ embryos fully restored adaxial AChR prepattern (Figure 1E). Thus, similar to its requirement in axonal pathfinding, the unplugged SV1 receptor, which lacks the Ig domains, is responsible for AChR prepattern, consistent with the idea that in vivo both processes share a common signaling mechanism.

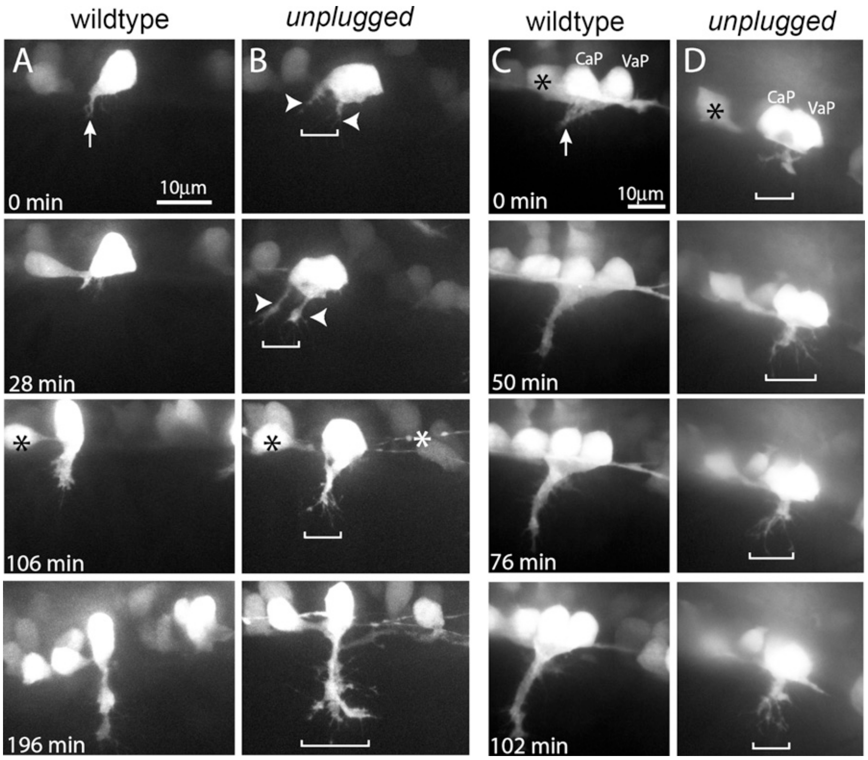

Figure 2. *unplugged* Restricts Navigating Growth Cones to a Central Muscle Zone

(A–D) Still images from time-lapse movies showing the initial migration of single CaP axons (A and B) or CaP/VaP pair axons (C and D) from the spinal cord into the myotome. Arrows point to the single wild-type CaP growth cone (A) and to the tightly fasciculated wild-type CaP/VaP growth cones (C). In contrast, unplugged CaP neurons form extensive filopodia and even multiple growth cones (arrowheads) that occupy a broader area (brackets, [B]). Similary, mutant CaP/VaP growth cones appear defasciculated and occupy a broader area compared to wild-type. Asterisks indicate interneurons also labeled by the Tg(Hb9:GFP) transgene.

unplugged/MuSK Restricts Growth Cone Migration to a Central Muscle Zone

We then asked if unplugged/MuSK co-ordinates axonal pathfinding and AChR prepatterning. While we had previously shown that in the zebrafish embryo unplugged/MuSK is critical for axonal guidance after the period of AChR prepatterning (Zhang and Granato, 2000), we decided to examine axonal pathfinding at an earlier stage during the period of AChR prepatterning. For this, we used the Hb9 transgenic line, in which motoneurons express GFP (Flanagan-Steet et al., 2005). We imaged pioneering wild-type and unplugged mutant growth cones, as they exited from the spinal cord and entered the muscle territory, traversing the central muscle zone. In wild-type embryos, the first motor growth cone to exit from the spinal cord is caudal primary (CaP), and in 50% of the hemisegments it is accompanied by variable primary (VaP), which tightly fasciculates with CaP (Eisen et al., 1990). Confocal-time lapse imaging confirmed that, once they exited from the spinal cord, wild-type CaP and CaP/VaP growth cones pioneered a tight and narrow path (n = 5 growth cones from five embryos; Figures 2A and 2C, Movie S1, and Eisen et al., 1986).

In contrast, unplugged CaP and VaP growth cones displayed aberrant growth cone morphologies as they traversed the muscle territory (Figures 2B and 2D and Movies S1 and S2; n = 8/8 growth cones from eight embryos). Frequently, unplugged CaP formed excessive filapodia, sometimes even multiple distinct and transient growth cones, which spread and occupied a much wider path no longer restricted to the muscle center (Figure 2B). Similarly, when CaP and VaP neurons pioneered the path simultaneously, their growth cones invaded lateral muscle territory that they usually avoid (Figure 2D and Movie S2). Because the unplugged/MuSK gene is only expressed and functions in muscle cells, we conclude that unplugged/MuSK-dependent cues produced by muscle cells confine growth cones to a narrow path in the central muscle region. Thus, live-cell imaging demonstrates that during pathfinding unplugged/MuSK limits the muscle territory accessible to growth cones, consistent with the idea that its primary role is to organize a common central muscle zone to which pioneering growth cones and the first AChR clusters are restricted.

The Noncanonical *wnt11r* Ligand Plays a Role in AChR Prepatterning and Axonal Guidance

We next asked which signaling pathway might activate the unplugged/MuSK receptor to organize a central muscle zone. We had previously shown that the unplugged^{p31CD} mutant allele carries a missense mutation that changes one of the ten conserved cysteines in the CRD, the Wnt-binding domain of frizzled receptors (Zhang et al., 2004). Unplugged/MuSK belongs to a small group of non-frizzled CRD-containing proteins, including the ROR receptors (Xu and Nusse, 1998), and RORs have recently been shown to directly interact through their CRD with Wnts (Hikasa et al., 2002; Oishi et al., 2003). Furthermore, Wnts play critical roles in synapse formation in the mammalian CNS, in Drosophila, and in C. elegans (reviewed in Speese and Budnik, 2007). One attractive idea is that Wnt signaling via the unplugged/MuSK receptor may induce the formation of a central zone along the anterior-posterior axis of developing muscle. We therefore reasoned that noncanonical Wnt family members known to induce such cellular polarity would be excellent candidates. Expression pattern analysis of published noncanonical Wnt genes during the relevant developmental window (17–24 hpf) identified several candidate Wnt genes, including pipetail (ppt)/wnt5a (Rauch et al., 1997), silberblick (slb)/wnt11(Heisenberg et al., 2000), and wnt11r (Matsui et al., 2005). Analysis of ppt/wnt5a and slb/wnt11 mutants did not reveal any axonal or synaptic defects (Figures S2A–S2C).

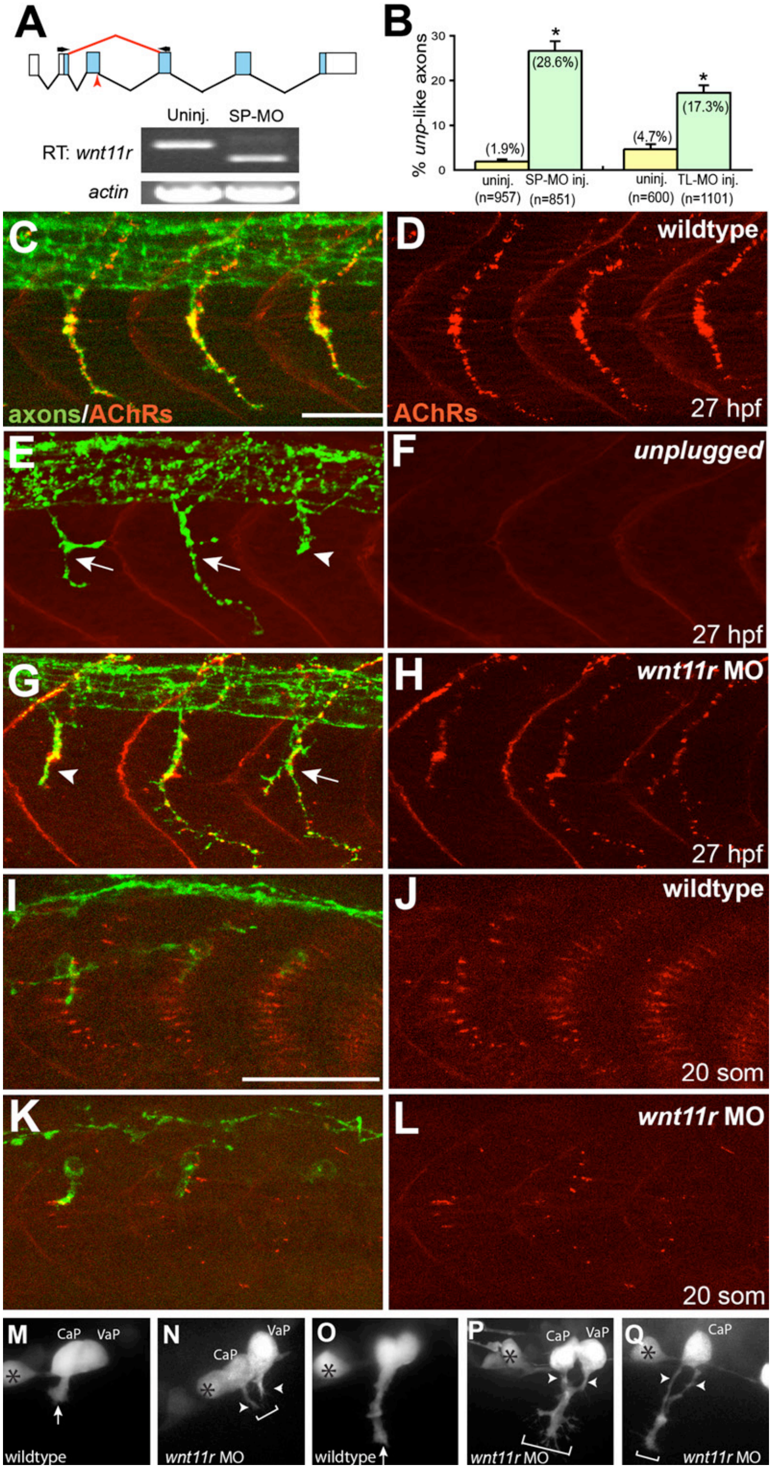

Figure 3. *wnt11r* Is Critical for Axonal Guidance and AChR Prepatterning

(A) The splice morpholino (SP-MO) targets the splice donor site of the wnt11r exon 3 (red arrow), and MO-induced aberrant splicing is shown in red. RT-PCR analyses of uninjected and wnt11r SP-MO injected embryos (arrows indicate the position of PCR primers).

(B) Quantification of wnt11r MOs injected embryos. TL-MO, translation initiation mopholino. Per embryo, 20 hemisegments were analyzed; n = hemisegments. Results are expressed as the mean of multiple injection experiments ± SEM, (*p < 0.001, t test).

(C–L) Wild-type, unplugged, and wnt11r MO injected embryos at 27 hpf (C–H) and at the 20 somite stage (I–L), stained for motor axons (znp-1, green) and AChR clusters (α-BTX, red). (E and F) In contrast to wild-type, unplugged embryos display characteristic stalling (arrowhead) and branches (arrows) at the choice point and lack all AChR clusters. (G and H) Injection of wnt11r MO causes unplugged-like axonal stalling (arrowhead), branching (arrow), and a strong reduction of AChR prepatterning (K and L). Note that the size and intensity of neural AChR clusters is reduced in wnt11r 27 hpf morphants (H).

(M–Q) Time-lapse images of Hb9-GFP-labeled wild-type (M and O) and wnt11r morphant CaP and VaP axons (N, P, and Q) as they exit from the spinal cord (M and N) and as they reach the somitic choice point (O–Q). Asterisks indicate the cell body of interneurons. (M and O) Wild-type CaP and VaP neurons extended one growth cone (arrow). Note the broad area (brackets) the two defasciculated wnt11r morphants CaP/VaP growth cones occupy (arrowheads in [N], [P], and [Q]), compared to wild-type (M and O).

Scale bars, 50 μm.

Analysis of wnt11r mRNA expression in 20 somite stage embryos revealed strong signals in the spinal cord and in the dorsolateral somites, just adjacent to unplugged/MuSK-expressing dorsal adaxial cells, consistent with previously published data (Figures S2D–S2G and Groves et al., 2005; Matsui et al., 2005). Thus, wnt11r is expressed at the right time and place to initiate unplugged/MuSK signaling in adaxial cells, at least in dorsal adaxial cells. We next tested the role of wnt11r, using a previously published translation-initiation-blocking morpholino (Matsui et al., 2005) and a newly designed splice-blocking morpholino. This second morpholino targets the exon 3 donor splice site, predicted to cause a frameshift-induced premature stop codon after amino acid 67 (Figure 3A; for details see Experimental Procedures). As determined by RT-PCR, injection of the splice-blocking morpholino caused an almost complete reduction of wnt11r transcript (Figure 3A). Knockdown of wnt11r using either of the two morpholinos did not affect specification, migration, or differentiation of adaxial muscle fibers (Figures S2J and S2K). Importantly, orientation of muscle fibers, which in chick embryos is thought to be controlled by a wnt/PCP pathway (Gros et al., 2008), was completely unaffected (Figures S2J and S2K). Instead, we observed two prominent phenotypes: axonal stalling and branching at 27 hpf (28% of hemisegments, n = 851, Figures 3B–3H), and a strong reduction of adaxial AChR prepattern (Figures 3I–3L). While AChR prepattern was severely affected, later developing neural AChR clusters developed, albeit their size was slightly reduced (Figures 3G and 3H). Finally, we also noticed a reduction of chondroitin sulfate proteoglycans (CSP) localization at the somite choice point (Figures S2H and S2I), identical to what we previously observed in unplugged/MuSK mutants (Zhang et al., 2004). Thus, morpholino knockdown of wnt11r causes unplugged/MuSK-like CSP, axonal, and AChR prepattern phenotypes.

To determine if wnt11r is also critical earlier to restrict motor growth cones to the central muscle zone, we imaged pioneering motor growth cones in wnt11r morpholino-injected Tg(Hb9:GFP) embryos. In these embryos, the number and position of GFP-positive motoneurons was indistinguishable from wild-type (Figures 3M–3Q; wnt11r: n = 4/6 growth cones from six morphants). However, as they entered the muscle, wnt11r morphant growth cones strayed away from the central zone and formed excessive filapodia (Figure 3M-Q). Thus, wnt11r morphants display AChR prepattern and axonal guidance defects identical to those observed in unplugged/MuSK mutants, suggesting that wnt11r acts through unplugged/MuSK.

Wnt11r Binds to Unplugged/MuSK

The similarity between the wnt11r morphant and the unplugged/MuSK mutant phenotypes suggested that both genes play roles in the same process. To test this, we first examined if both genes interact genetically. For this, we injected a suboptimal dose of wnt11r morpholino into wild-type embryos, which induced unplugged-like axonal defects in 13.3% of hemisegments (n = 977 hemisegments; Figure S3A). unplugged heterozygous embryos do not display any phenotypes, and injection of these embryos with a suboptimal wnt11r MO dose significantly increased the number of axonal phenotype to 23.3% (n = 1179 hemisegments; Figure S3A). Moreover, using the same approach, we also observed an increase in AChR prepatterning defects, demonstrating that wnt11r and unplugged/MuSK interact genetically (Figures S3B and S3C).

The genetic interaction results further suggested that both genes play roles in the same process. One attractive hypothesis first suggested by Burden et al. is that secreted Wnt proteins directly bind the unplugged/MuSK receptor through its CRD (Burden, 2000). We therefore examined whether Wnt11r protein can physically associate with the extracellular region of unplugged. We initially focused on the unplugged SV1 isoform, because it is required for axon guidance and AChR prepatterning in vivo. GST-Unplugged fusion proteins, consisting of the unplugged SV1 extracellular domain tagged with GST at the N terminus (GST-SV1-ECD), were coupled to glutathione-Sepharose and then mixed with conditioned medium containing secreted Wnt11r-FLAG. Wnt11r proteins bound to GST-Unplugged were then detected by anti-FLAG immunoblotting. As shown in Figure 4A, Wnt11r-FLAG binds to the extracellular Unplugged SV1 region in vitro, suggesting that the extracellular domain of Unplugged associates with Wnt11r.

We next examined the physical association between Wnt11r and Unplugged in more detail. Since Unplugged/MuSK proteins contain the CRD known to function as the Wnt-binding sites of Frizzled proteins, we tested whether the unplugged CRD is required for Wnt11r binding. Myc-tagged full-length Unplugged SV1 (Unplugged SV1-myc) and myc-tagged Unplugged with the CRD deleted (unplugged SV1 ΔCRD-myc) were cotransfected into 293T cells with FLAG-tagged Wnt11r (Wnt11r-FLAG). Cell lysates were processed for immunoprecipitation with anti-Myc antibody followed by western blotting with anti-FLAG antibody. Wnt11r bound to full-length Unplugged SV1 but not to Unplugged SV1ΔCRD, demonstrating that the Unplugged CRD is required for Wnt11r binding (Figure 4B). The unplugged FL isoform, which is similar to mammalian MuSK, also binds Wnt11r, albeit more weakly (Figure S4A).

To determine if Wnt11r binds Unplugged/MuSK in vivo, we examined Wnt11r-FLAG binding in embryos. For this, we affinity purified Wnt11r-FLAG protein from supernatant of transfected 293T cells and injected the soluble protein into the yolk sac of 15 somite stage live embryos (just prior to the onset of AChR prepatterning). The injected protein is transported in the extracellular spaces throughout the entire embryo and is exposed to the surface of all cells (Lefebvre et al., 2004). In 20 somite stage wild-type embryos, we detected Wnt11r-FLAG binding on adaxial cells, coinciding exclusively with the unplugged/MuSK expression domain (Figure 4C, compare to Figure S1D). In unplugged mutant embryos, binding of Wnt11r-FLAG was completely abolished (Figure 4D). Thus, together our results demonstrate that unplugged/MuSK has properties of a receptor for Wnt proteins.

Finally, we asked if wnt11r plays a permissive or an inductive role in AChR prepatterning by testing if wnt11r overexpression is sufficient to induce ectopic AChR prepattern. Injection of mRNA encoding for wnt11r or unplugged SV1 into wild-type embryos revealed no difference in AChR prepattern (Figures 4E, 4F, and 4I). In contrast, coinjection of both wnt11r and unplugged SV1 mRNAs induced ectopic AChR clusters (Figures 4G and 4I). To tested whether the unplugged CRD is required in vivo for wnt11r-induced AChR prepatterning, we also coinjected wnt11r and unplugged SV1ΔCRD (lacking the Wnt-binding domain), which did not increased AChR prepattern (Figures 4H and 4I). Thus, our results show that unplugged/MuSK and wnt11r are both mutually required for induction of the AChR prepattern, consistent with a ligand-dependent mode of action.

Blocking *disheveled* Function in Adaxial Cells Causes *unplugged*-like Phenotypes

Next, we asked if signaling downstream of wnt11r and unplugged/MuSK requires the obligate Wnt intracellular effector dishevelled. Recent studies have shown that the kinase domain of MuSK interacts with dishevelled through its DEP domain, critical for activation of the noncanonical Wnt pathway (Luo et al., 2002). We first used the yeast two-hybrid system to confirm that the zebrafish Unplugged kinase domain interacts with zebrafish Dishevelled (Figure S5A). We then used a truncated form of dishevelled, XDsh−DEP+, shown to specifically block noncanonical Wnt signaling in flies, Xenopus, and zebrafish (Axelrod et al., 1998; Heisenberg et al., 2000; Wallingford et al., 2000). To avoid interference with earlier developmental processes, we used the smyhc1 promotor to target expression of myc-tagged XDsh−DEP+ specifically to adaxial cells, and then generated transient transgenic embryos expressing Myc-XDsh−DEP+ in a small, stochastic subset of adaxial cells.

Analysis of transient transgenic embryos revealed unplugged-like axonal phenotypes in somitic segments expressing

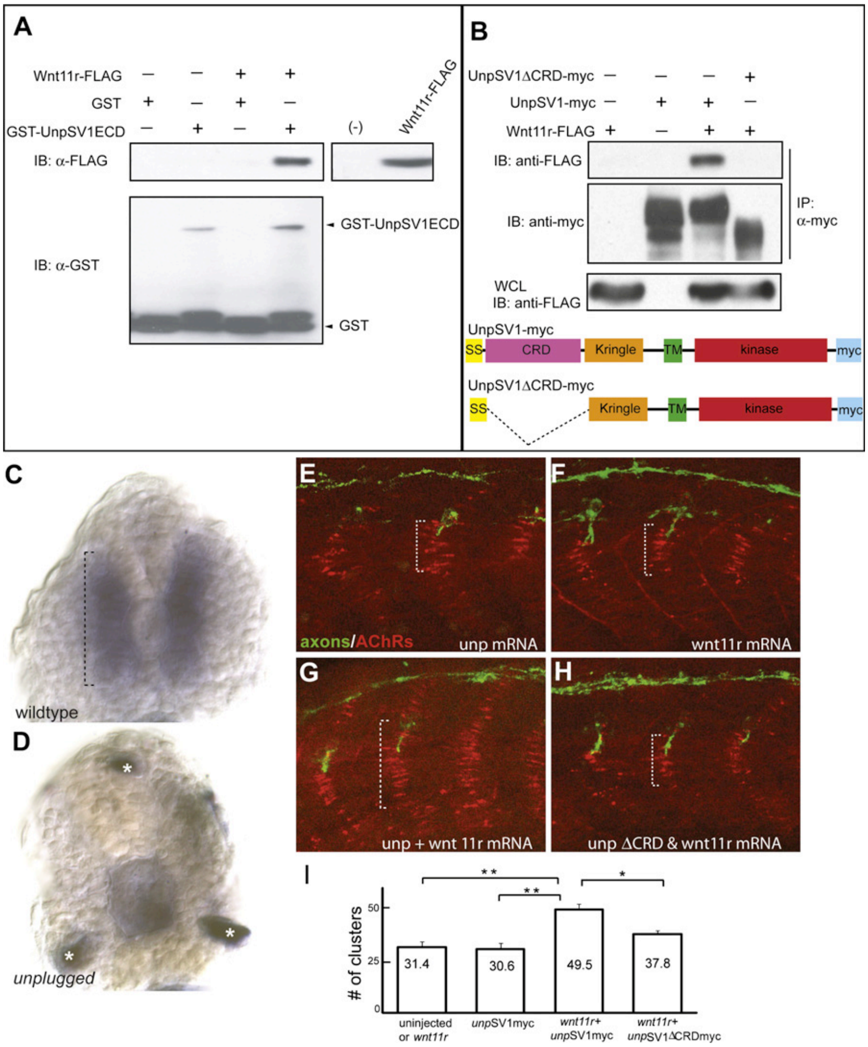

Figure 4. Wnt11r Binds to UnpSV1 and Overepressions of *wnt11r* and *unpSV1* Increase Prepatterning

(A) Binding of Wnt11r to the extracellular domain (ECD) of UnpSV1 in vitro. GST-UnpSV1ECD fusion proteins, coupled to glutathione sepharose, were mixed with conditioned media containing secreted Wnt11r-FLAG. Amounts of GST-UnpSV1 and Wnt11r-FLAG used in the analysis were assessed by anti-GST (lower panel) and anti-FLAG (right panel) immunoblotting (IB), respectively. Amounts of Wnt11r-FLAG bound were evaluated by anti-FLAG IB (upper panel).

(B) Coimmunoprecipitation of UnpSV1 with Wnt11r in 293T cells. 293T cells were cotransfected with Wnt11r-FLAG and UnpSV1-myc or its CRD deletion mutant. Whole-cell lysates (WCL) were subjected to anti-FLAG IB to determine the expression of Wnt11r-FLAG (lower panel). The UnpSV1-bound Wnt11r was assessed by IB of the anti-myc immunoprecipitate (upper panel). Schematic diagrams of constructs used in the experiments. SS, signal sequence.

(C and D) Cross-sections of 20 somite stage embryos injected with purified Wnt11r-FLAG protein. (C) In wild-type embryos, Wnt11r binds to adaxial cells as highlighted by the brackets. Binding is abolished in unplugged mutants(asterisks in [D] mark nonspecific staining).

(E–H) Wild-type embryos were injected with mRNAs as indicated. The domain of AChR prepatterning (brackets) was expanded in embryos coinjected with wnt11r and unpSV1myc mRNAs and was dependent on the CRD domain (G and H).

(I) Co-overexpression of wnt11r and unpSV1 significantly increases the number of prepatterned clusters/hemisegment (n = 5–18 hemisegments per bar, average = 10). Results are expressed as the average of different injection experiments (t test, $^{**}p < 0.01$, $^{*}p < 0.05$). Amounts of mRNA (ng/embryo): wnt11r-FLAG, 0.3; SV1myc, 0.5; SV1⊿CRDmyc, 0.5. AChR cluster size distribution was not altered.

Myc-Dsh-DEP+ in dorsal but not in ventral adaxial cells (Figures 5A and 5B and data not shown), consistent with the observation that unplugged function is required only in dorsal adaxial cell to guide motor axons (Zhang and Granato, 2000). Furthermore, the frequency of pathfinding defects, up to 36%, correlated with the number of Myc-Dsh-DEP+ positive dorsal adaxial cells (Figures 5C and 5D). Analysis of the AChR prepattern revealed that expression of Myc-Dsh-DEP+ in individual adaxial fibers coincided with a marked reduction of clustered AChRs (Figures 5E–5F'). Finally, expression of Myc-Dsh-DEP+ did not affect specification, migration, or differentiation of adaxial muscle fibers (Figures S5B and S5C), suggesting that the AChR and axonal phenotypes are the primary result of blocking noncanonical Wnt signaling. Thus, blocking Wnt downstream signaling in adaxial cells recapitulates two main phenotypes characteristic for unplugged/MuSK mutants, consistent with the idea that cell-autonomous, noncanonical Wnt signaling in adaxial cells is critical for axonal guidance and AChR prepatterning.

Synapses Form in the Absence of AChR Prepattern

Our results show that unplugged/MuSK and wnt11r play critical roles in initiating the AChRs prepattern. These prepatterned AChR clusters can be incorporated into neuromuscular junctions, but is AChR prepattern essential for synapse formation? To answer this question, we generated multiple inducible unplugged/MuSK transgenic lines, in which the heat-shock protein 70 (hsp70l) promoter (Halloran et al., 2000) drives ubiquitous expression of myc-tagged unplugged FL or myc-tagged unplugged SV1. We then crossed these lines into unplugged/MuSK null mutants and confirmed that in the absence of heat-shock treatment Tg(hsp70l:unplugged FL-myc); $unplugged^{(br307/br307)}$ or Tg(hsp70l:unplugged SV1-myc); $unplugged^{(br307/br307)}$ embryos lacked all AChR prepattern and neuromuscular synapses (Figures S6A–S6D).

We then used continuous heat-shock treatment to induce expression of Tg(hsp70l:unplugged FL-myc) or Tg(hsp70l: unplugged SV1-myc) in $unplugged^{(br307/br307)}$ embryos, starting several hours before the first AChR clusters become detectable.

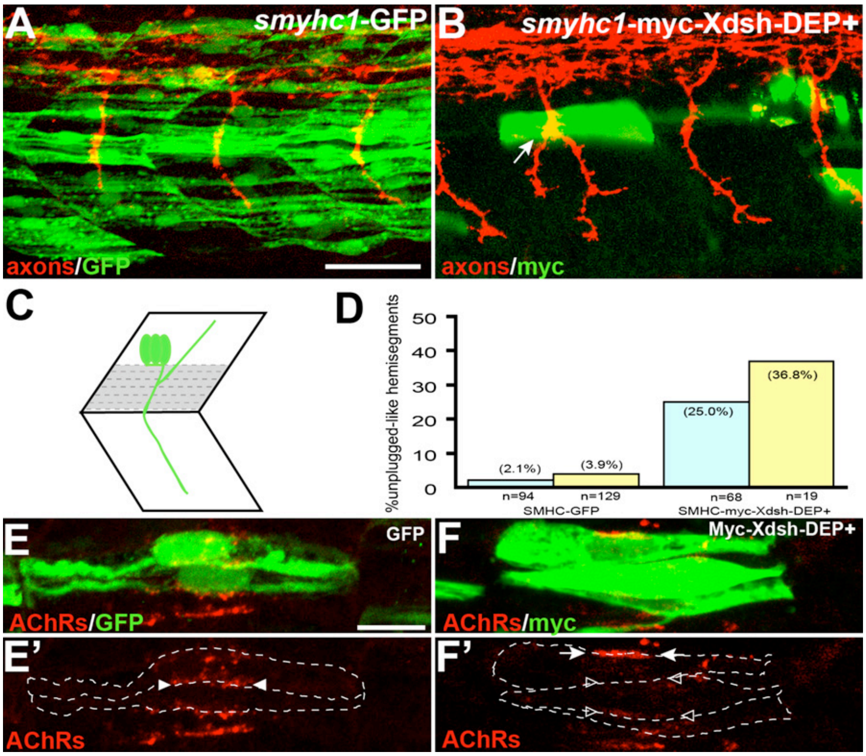

Figure 5. Inhibition of the Noncanonical Dsh Pathway in Adaxial Fibers

(A) Stochastic expression of Tg(smyhc1:GFP) in adaxial muscle (green) does not affect motor axons (red).

(B) Expression of Tg(smyhc1:myc-XDsh-DEP+) (green) in adaxial fibers dorsal to the choice point causes unplugged-like pathfinding defects (arrow).

(C) Location of the dorsal six or seven adaxial cells (in gray) used for scoring.

(D) Analysis of axonal phenotypes. n, hemisegments; blue, hemisegments with two adaxial cells expressing the transgene; yellow, hemisegments with three or more adaxial cells expressing the transgene.

(E–F') Confocal images of adjacent adaxial muscle pioneers expressing the smyhc1-GFP or smyhc1-myc-Xdsh-DEP+ transgene. Only AChR clusters between two adjacent transgene-positive adaxial cells were analyzed (outlined by dashed lines). Tg(smyhc1:GFP) expressing adaxial cells form AChR clusters (arrowheads in [E']), while Tg(smyhc1:myc-XDsh-DEP+ expression disrupts AChR clusters between transgene expressing cells ([F'], open arrowhead); note that this does not affect adjacent, nontransgenic cells that formed normal AChR clusters ([F'], arrows). For each transgene, four embryos with GFP or Myc-Dsh-DEP+ positive adaxial cells were analyzed. Prepatterned clusters were reduced in all Myc-Dsh-DEP+ expressing embryos.

Scale bars: (A) 50 μm, (E) 10 μm.

Heat-shock treatment (see Experimental Procedures for details) was applied until 27 hpf, at which time point growth cones have migrated past the somitic choice point and have formed neural synapses. Analysis of these embryos revealed that unplugged SV1-myc transgene expression completely restored AChR prepattern but failed to induce neural AChR clusters (Figures 6A–6D), consistent with previous observations that the Ig domains absent in the SV1 isoform ectodomain are critical for agrin responsiveness leading to the formation of neuromuscular synapses (Zhou et al., 1999). As predicted, expression of the unplugged FL-myc transgene in unplugged embryos almost completely restored neural AChR clusters (Figures 6E and 6F). In these "rescued" embryos, AChR cluster size was slightly reduced, but most AChR clusters were precisely apposed to axonal varicosities, identical to wild-type synapses (Figures S6E–S6F'). This confirms that the extracellular Ig domains of the unplugged/MuSK receptor are critical for the late stage of synapse formation, when AChRs become incorporated into functional neuromuscular synapses.

However, we noticed that heat-shock-treated Tg(hsp70l:unplugged FL-myc; unplugged$^{(br307/br307)}$ embryos displayed very little or no AChR prepattern (Figures 6G and 6H). We confirmed the absence of detectable adaxial AChR prepattern in three independent transgenic lines, suggesting that this was not due to the influence of chromatin neighboring the transgene integration site. Furthermore, western blot analysis of Tg(hsp70l:unplugged FL-myc); unplugged$^{(br307/br307)}$ embryos showed that heat-shock treatment induced high levels of myc-tagged protein, comparable to the levels in Tg(hsp70l:unplugged SV1-myc); unplugged$^{(br307/br307)}$ embryos (Figures S6H). Finally, expression of unpluggedFL under the control of the adaxial-specific promoter Tg(smyhc1:unpluggedFL-myc) in unplugged $^{(br307/br307)}$ embryos also failed to restore adaxial AChR prepattern (Figures S4B and S4C). These experiments indicate a potential negative role for the Ig domains on the AChR prepattern, but more importantly suggest that in vivo neural synapses can form in the absence of prepatterned AChRs.

To exclude the possibility that in heat-shock-treated Tg(hsp70l:unplugged FL-myc); unplugged$^{(br307/br307)}$ embryos adaxial AChR prepattern was present but not detectable, e.g., due to small AChR cluster size, we repeated the experiment but started heat-shock treatment after the time period of AChR prepatterning, when motor axons have extended well into the periphery (26 somite stage, ~22 hpf). Analysis of these embryos at 27 hpf revealed the characteristic unplugged axonal defects and the presence of neural AChR clusters (Figures 6I and 6J). Although these "rescued" AChR clusters were abundant, they were smaller in size and less precisely aligned with the axons when compared to wild-type (Figures 6I and 6J and Figures S6G and S6G'). Nonetheless, heat-shock-treated Tg(hsp70l:unplugged FL-myc; unplugged$^{(br307/br307)}$) embryos were fully motile, suggesting that AChR clusters represent functional neuromuscular synapses. Thus, functional neuromuscular synapses can develop in the absence of AChR prepattern, and in the absence of unplugged/MuSK function during the early, nerve-independent phase. This suggests that nerve-muscle interactions

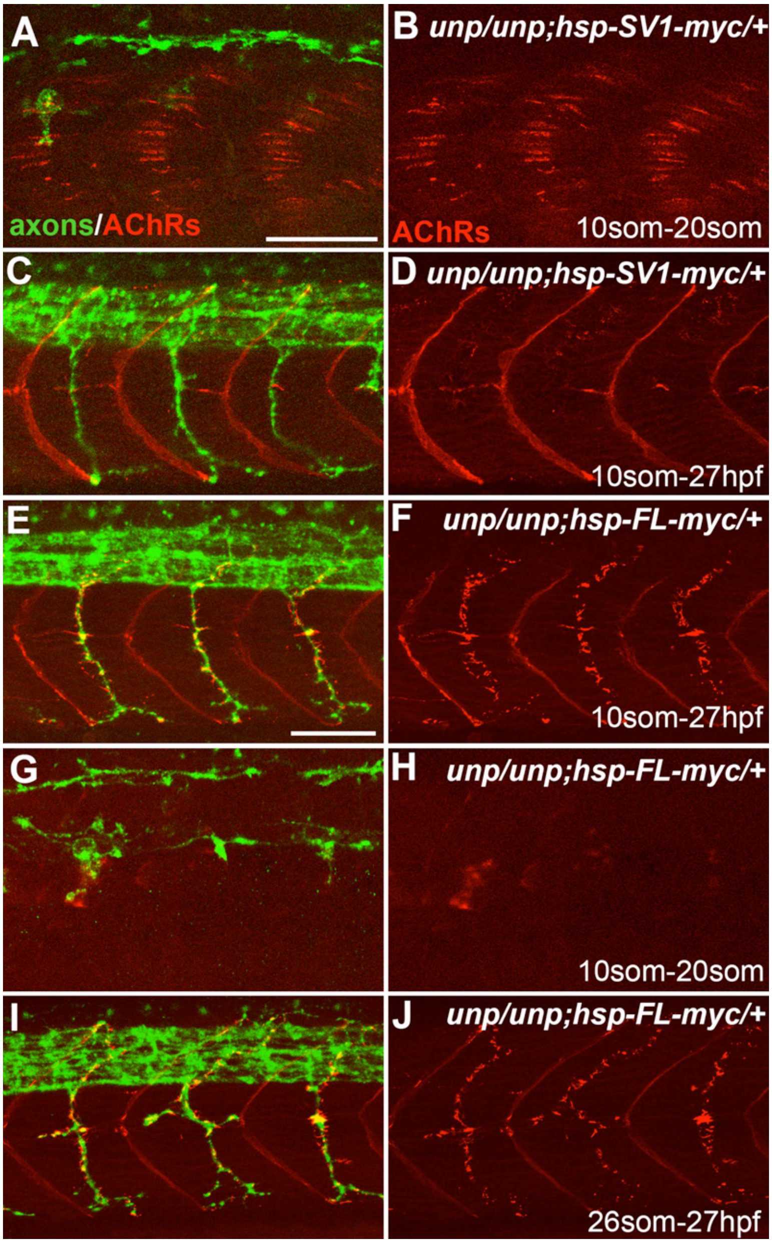

Figure 6. Neuromuscular Synapses Form in the Absence of AChR Prepattern

Twenty-somite stage (A, B, G, and H) or 27 hpf (C–F, I, and J) embryos after heat-shock treatment.

(A and B) Tg(hsp70l:UnpSV1-myc;unplugged) embryos received heat shock from the 10 to 20 somite stage, which rescued AChR prepattern.

(C and D) Similar heat-shock treatment (10 somite to 27 hpf) also restored motor axon pathfinding, but not neuromuscular synapses.

(E and F) The same heat-shock treatment rescued motor axons and neuromuscular synapses in Tg(hsp70l:UnpFL-myc; unplugged) embryos.

(G and H) In contrast, heat shock between the 10 and 20 somite stage failed to rescue AChR prepattern in Tg(hsp70l:UnpFL-myc;unplugged) embryos.

(I and J) Heat-shock treatment of same embryos between the 26 somite stage and 27 hpf, i.e., after the time period of prepatterning, was sufficient to rescue neuromuscular synapses.

Scale bars, 50 μm.

at the late phase of synapse formation can compensate for the absence of an AChR prepattern and that these interactions are sufficient to generate neuromuscular synapses in vivo.

DISCUSSION

The Role of Wnt Signaling in Synapse Formation

Recent studies in *C. elegans*, *Drosophila*, and in the mammalian CNS have revealed critical roles for Wnt ligands in synapse formation (Hall et al., 2000; Klassen and Shen, 2007; Packard et al., 2002). At the mammalian neuromuscular junction, the precise role of Wnt signaling is less clear. In vitro, Wnt-1 has no influence on AChR clustering (Luo et al., 2002) but can regulate MuSK expression in cultured myotubes (Kim et al., 2003). Recent studies using cultured myotubes show that Wnt3 increases agrin-dependent AChR clustering (Henriquez et al., 2008), and several downstream components of the Wnt pathway, including β-catenin, Dishevelled, APC, PAK, and JNK have also been implicated in this process (Luo et al., 2002; Wang et al., 2003; Zhang et al., 2007). More recently, the low-density lipoprotein receptor-related protein 4 (LRP4) whose extracellular domain is similar to the Wnt coreceptor LRP5/6 proteins has been shown to function as a MuSK coreceptor binding nerve-released Agrin, and thus promoting neural AChR clusters (Kim et al., 2008; Zhang et al., 2008). Interestingly, LRP4 is also required for AChR prepattern and the accumulation of MuSK protein at presumptive synapses, supporting a role for Wnt signals in the early phase of NMJ development (Weatherbee et al., 2006). However, it has remained unclear if and which Wnt ligand(s) can activate the early, nerve-independent AChR prepattern.

Our results provide four compelling lines of evidence that Wnt ligands signal through unplugged/MuSK to initiate the early, nerve-independent phase of synapse development. First, morpholino-mediated reduction of wnt11r causes severe AChR prepatterning defects, as well as unplugged-like axonal defects already as growth cones navigate toward the AChR prepattern. Second, wnt11r and unplugged/MuSK interact genetically, suggesting that they function in the same pathway. Third, in vitro Wnt11r binds to Unplugged in a CRD-dependent manner, and in vivo Wnt11r-FLAG-binding sites precisely outline the unplugged/MuSK expression domain, i.e., adaxial muscle, in a unplugged-dependent fashion, suggesting that Unplugged/MuSK has the properties of a wnt11r receptor. Fourth, blocking the disheveled-dependent noncanonical Wnt pathway in adaxial cells also causes defects in AChR prepatterning and axonal pathfinding.

Together, these data suggest that in response to Wnt ligands muscle cells enable an unplugged/MuSK signaling cascade that restricts growth cones and AChR prepattern to a common muscle zone.

During Drosophila NMJ development, Wnt-1 (Wg) is secreted presynaptically to regulate synapse development (Speese and Budnik, 2007), raising the question of the relevant Wnt11r source in the zebrafish embryo. Although wnt11r is expressed in the spinal cord, it is unlikely that motor nerves are the source of wnt11r. AChR prepattern is visible well before motor growth cones approach (Figure S1), and in mammals AChR prepatterning has been shown to be independent of nerve contacts and signals (Lin et al., 2001; Yang et al., 2001). Based on its spatial mRNA expression, wnt11r is likely secreted by cells in the dorsolateral somites (Figures S2D–S2F), adjacent to premigratory dorsal adaxial cells in which unplugged function is required (Zhang and Granato, 2000). Interestingly, Wnt11r secreted from these dorsolateral somitic cells induces AChR prepattern on ventral adaxial cells (Figure 3K), at a distance of about ten cell diameters, reminiscent of the well-studied long-range action of Drosophila wingless (Zecca et al., 1996). While Wnt proteins are hydrophobic and probably membrane associated, after secretion, Wnts can diffuse at a rate of up to 50 μm in 30 min and can act as long-range signals up to 20 cell diameters away (Strigini and Cohen, 2000; Wodarz and Nusse, 1998). More recently, it has become clear that long-range activation is likely mediated by Wnt proteins uniquely packed for long-range signaling (reviewed in Bartscherer and Boutros, 2008). In the embryo, Wnt11r-FLAG binds to dorsal and ventral adaxial muscle (Figure 4), consistent with the idea that wnt11r-dependent formation of AChR prepattern in ventral adaxial cells might be mediated by a long-range signaling mechanism.

However, given the presence of residual AChR prepatterning and low penetrance of axonal pathfinding defects in wnt11r morphants, it is also possible that additional Wnt ligands, possibly expressed in other tissues, activate the unplugged receptor in more ventral adaxial cells. Although our understanding of how Wnt signals direct activation of unplugged/MuSK is only beginning to emerge, together our data provide compelling evidence that during the early phase of synapse formation, Wnt signals through the unplugged receptor organize a central muscle zone to which navigating motor growth cones and nascent AChR prepattern are confined.

AChR Prepattern Is Dispensable for NMJ Formation

In zebrafish, prepatterned AChR clusters can be incorporated into prospective neuromuscular synapses upon contact with motor axons (Flanagan-Steet et al., 2005; Panzer et al., 2006), and in mice aneural AChR clusters per se are not required for synapse formation (Lin et al., 2008; Vock et al., 2008), but it has remained unclear if AChR prepattern itself is essential for synapse formation. Our results demonstrate that activation of unplugged/MuSK in unplugged mutants after the period of AChR prepatterning results in the unexpected presence of almost wild-type-like, functional neuromuscular synapses. This demonstrates that AChR prepatterning is dispensable for subsequent synapse formation. However, we cannot exclude the possibility that, during normal development, AChR prepatterning facilitates or serves as an initial scaffold for future synapses, as pre-existing AChR clusters are incorporated into neuromuscular junctions (Flanagan-Steet et al., 2005; Panzer et al., 2005). It is also possible that high levels of unplugged/MuSK protein from the hsp70l transgene compensate for the absence of prepatterned AChRs. For example, expression of constitutively active MuSK leads to self-aggregation, and these aggregates colocalize with AChR clusters, even in the absence of agrin (Jones et al., 1999). Independent of how "late" unplugged/MuSK activation induces neuromuscular synapses, our results provide insights to a longstanding question. In the now widely accepted "myocentric" model, the muscle determines the future site of synaptogenesis. It has also been long known that motoneurons can form synapses with cultured muscle cells lacking an AChR prepattern, suggesting that such prepattern might not be essential (Anderson and Cohen, 1977; Frank and Fischbach, 1979). Our results demonstrate that, in the embryo, functional synapses can develop in the complete absence of the initial AChR prepattern and suggest that, during the late phase of synapse formation, synapses form de novo at sites where the nerve releases Agrin to locally activate MuSK, or possibly by local MuSK autoactivation (Kim and Burden, 2008; Lin et al., 2008). Importantly, while AChR prepattern is dispensable, e.g., by late expression of unplugged/MuSK, the organization of a central muscle zone is essential to restrict growth cones, as "late" unplugged/MuSK expression fails to rescue the axonal pathfinding defects (Figure 6). Thus, the central zone determines the muscle territory accessible to motor axons, and thereby the sites of neuromuscular synapses.

The Role of *unplugged*/MuSK in Synapse Formation

What is the role of unplugged/MuSK in presynaptic development? In $MuSK^{-/-}$ mice, nerve processes are not restricted to the central region of the muscle, but are present throughout the muscle (DeChiara et al., 1996). This exuberant axonal growth has been attributed to the absence of MuSK-dependent muscle-derived signals, which normally stop axonal growth and induce presynaptic differentiation (DeChiara et al., 1996), but more recent analyses reveal the presence of axonal branching before the formation of AChR clusters (Lin et al., 2008). While at later stages unplugged/MuSK mutant embryos also display excessive branching (Zhang and Granato, 2000), our time-lapse analysis reveals dramatic defects earlier during axonal pathfinding (Figure 2). Like mammalian MuSK, unplugged expression is undetectable in motoneurons, and chimera analyses have shown that unplugged/MuSK functions in adaxial muscle to guide motor axons (Zhang and Granato, 2000). Thus, already very early on unplugged/MuSK-dependent, muscle-derived signals restrict growth cones to the central muscle zone. This raises the possibility that the later observed exuberant axonal growth is a consequence of the earlier guidance defects, although we cannot exclude the possibility that unplugged/MuSK provides several independent signals.

Our analyses reveal identical guidance and AChR defects in wnt11r morphants, and in combination with in vitro binding data this suggests that wnt11r activates unplugged/MuSK to organize a central muscle zone, thereby confining pre- and postsynaptic processes to a common, narrow domain. Intriguingly,

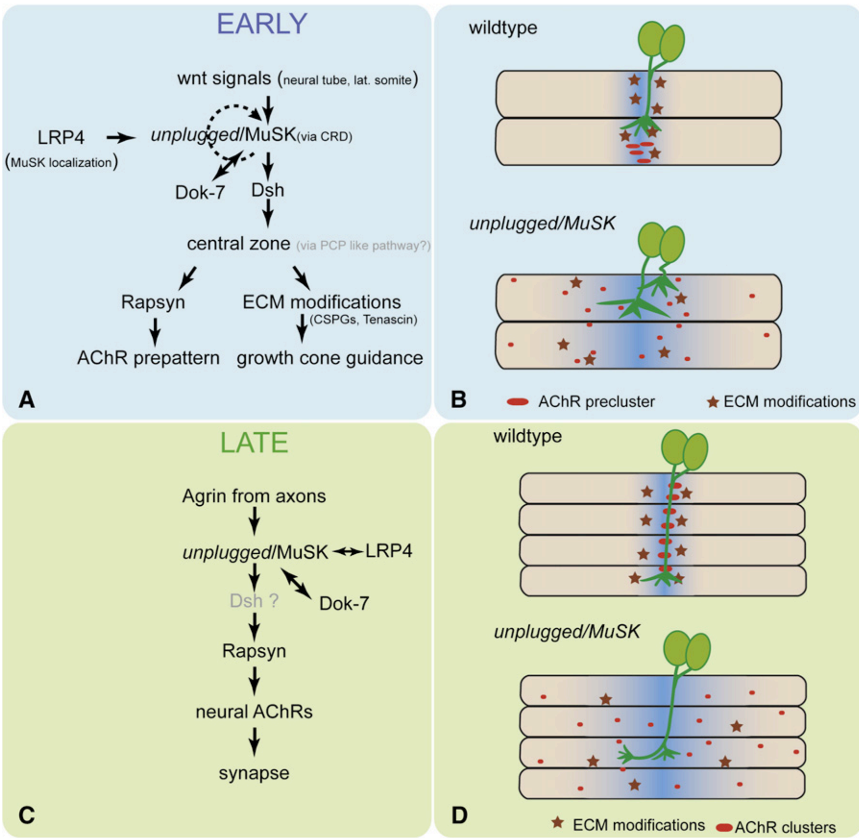

Figure 7. *unplugged*/MuSK Signaling during Synapse Formation

Signaling during the early (A and B) and late (C and D) phase of neuromuscular synapse formation.

(A and B) Early during synapse formation, Wnt signals act through unplugged/MuSK receptor to establish a central muscle zone, possibly through a Dsh-dependent, PCP-like pathway. LRP4 is essential for MuSK localization, and Dok-7 for MuSK activation (Okada et al., 2006). Ligand-dependent unplugged/MuSK activation may rapidly become ligand independent. One branch of this pathway requires rapsyn to cluster AChRs (red ovals in [B]) in a central prepattern, while, through a rapsyn-independent mechanism, e.g., modifications of the ECM components (dark red stars in [B]), growth cones are restricted to the central zone. In the absence of Wnt or unplugged/MuSK, rapsyn is not activated, and thus AChRs are dispersed throughout the muscle, and navigating growth cones extend into lateral muscle territory. Blue shades indicate the central zone.

(C and D) During the late phase, nerve-derived agrin signals through unplugged/MuSK and LRP4 to recruit rapsyn, which stabilizes neural AChRs and promotes synapse development. (D) In the absence of unplugged/MuSK, rapsyn is not recruited, and thus AChR cluster are not stabilized in the central zone. Absence of unplugged also causes rapsyn-independent pathfinding defects, possibly through the lack of ECM modifications. Note that in the absence of a central muscle zone at the early stages, no AChR prepattern forms but that local agrin secretion from the axon and late expression of unplugged/MuSK appears sufficient to induce neural AChRs and subsequently functional synapses.

only overexpression of Wnt11r and Unplugged induces ectopic AChRs, suggesting that wnt11r by itself is not sufficient to induce AChR prepatterning (Figure 4), but that additional, wnt11r-independent mechanisms, e.g., to localize the unplugged/MuSK receptor, are also critical. Based on our data, we propose a model in which, during the early phase of synaptogenesis, Wnt activates via unplugged/MuSK a dishevelled signaling pathway in muscle, similar to the planar cell polarity pathway to define the position of subcellular components along the anterior-posterior axis (Figures 7A and 7B). We propose that one branch of this pathway acts through rapsyn to accumulate AChR clusters to the central zone, thereby generating an AChR prepattern. This is consistent with the requirement of rapsyn in mouse and fish, as in its absence the AChR prepattern fails to form (data not shown and Lin et al., 2001). Rapsyn is not required for axonal guidance and presynaptic development (Zhang et al., 2004), while dishevelled is, suggesting a second, rapsyn-independent branch, downstream of dishevelled, to confine presynaptic growth (Figure 7A). Such a rapsyn-independent branch is also supported by live imaging, demonstrating that AChR clusters per se are not required for growth cone guidance (Panzer et al., 2005).

How does unplugged/MuSK signaling restrict growth cones to the central muscle zone? We have previously shown that unplugged/MuSK mutants lack a specific expression domain of two extracellular matrix (ECM) components along the anterior and posterior boundaries of the central muscle zone (Schweitzer et al., 2005; Zhang et al., 2004). This is highly significant because both components are produced by adaxial cells and because only their adaxial expression domain is altered in unplugged/MuSK mutants but not in other motor axon guidance mutants examined (Schweitzer et al., 2005; Zhang et al., 2004). Both of these ECM components, tenascin and chondroitin sulfate proteoglycans (CSP), have been implicated in axonal repulsion (Becker et al., 2003; Masuda et al., 2004), and here we show that wnt11r morphants also exhibit defects in CSP localization (Figure S2), consistent with a model by which these and/or additional wnt11r and unplugged/MuSK-dependent ECM modifications may restrict the axonal path of navigating growth cones to the central muscle zone.

Together, our data suggest a compelling model for the role of unplugged/MuSK in the early, nerve-independent phase of synapse formation (Figures 7A and 7B), preceding the better understood nerve/agrin-dependent late phase (Figures 7C and 7D). We propose that unplugged/MuSK engages a dishevelled-dependent signaling pathway in muscle cells to organize a central muscle zone, essential to confine navigating motor growth cones and nascent AChR prepattern to the center of muscle fibers. More importantly, we also propose that this process is initiated—at least in part—by Wnt signals. We identify wnt11r as a potential unplugged/MuSK ligand, but because of the incomplete penetrance of the wnt11r morphant phenotype, it is possible that additional, functionally redundant ligands and/or compensatory mechanisms exist. For example, Kim and Burden have recently proposed an elegant model by which ligand-independent MuSK activation in the mouse embryo is sufficient for AChR prepattern and presynaptic development (Kim and Burden, 2008). We propose that this complex process is initially ligand dependent—as overexpression of only wnt11r and unplugged/MuSK induces ectopic AChR clusters—but that it might rapidly become ligand independent due to a positive feedback loop (Jones et al., 1999; Moore et al., 2001). Elevating MuSK expression in early myofibers even slightly above the endogenous levels, as was done in the Kim and Burden study, may bypass the initial ligand dependency we observe. Alternatively, species-specific differences dictated by anatomical and/or developmental restrictions, such as muscle fiber length and speed of NMJ formation, may account for divergent mechanisms of unplugged/MuSK activation. Nonetheless, our results provide evidence that Wnt ligands are critical for initiating synapse formation and that Wnts can bind the unplugged/MuSK receptor. We propose that Wnt stimulation engages a dishevelled-dependent signaling cascade to establish polarity within the plane of the muscle, thereby registering AChR clusters with advancing growth cones, possibly through a mechanism reminiscent of the planar polarity pathway.

EXPERIMENTAL PROCEDURES

Whole-Mount Immunocytochemistry, wnt11r-FLAG In Vivo Staining

Embryos were fixed and stained as described in Zeller et al. (2002). For labeling of AChRs, embryos were permeabilized in 1 mg/ml collegenase (Sigma) in phosphate buffer for 6–8 min, rinsed in 1xPBS and incubated with Alexa Fluor-conjugated α-bungarotoxin (Molecular Probes, Eugene, OR) as described by Lefebvre et al. (2004). Antibodies and dilutions were used as follows: znp-1 (1:200, DSHB), SV2 (1:50, DSHB), myc (9E10, 1:1000, Covance), Prox1 (1:200), F59 (1:20, DSHB), and anti-chondroitin sulfate (CS56, 1:200, Sigma). Embryos were imaged with LSM510 (Zeiss) and LCS (Leica) confocal microscopes.

Wnt11r-FLAG was affinity purified from transfected HEK cells and injected into live embryos as previously reported for αBTX (Lefebvre et al., 2004) and detailed in the Supplemental Data section.

Quantification of AChR Clusters

Confocal images were projected into a single plane and converted to a 16 bit image using Metamorph. A region of interest was drawn around the border of each somitic segment. AChR clusters were counted using the "count nuclei" function, with the minimum/maximum length set to 5/100 pixels, respectively, and a minimum average intensity of 60 above background. The results were exported to Microsoft Excel for statistical analysis.

Morpholino and mRNA Injections

Three to four nanograms of wnt11r translation-blocking MO (wnt11r TL-MO) (Matsui et al., 2005) were injected into one-cell embryos. A splice-blocking MO (wnt11r SP-MO, 5′TTTTTCTCAGTAACTCACCTCGTTC3′) was designed against the splice donor site of exon 3. Six to seven nanograms of wnt11r SP-MO were injected into the embryos at one-cell stage. For RT-PCR analysis, cDNA templates were synthesized from five 24 hpf embryos. PCR primers were 5′-TCCTCACATTCCTGCTCCTGTC-3′ (forward) and 5′-TCTTCATCTT CATTGGGGCATC-3′ (reverse). mRNA was in vitro transcribed from linearized constructs using SP6 mMessage mMachine Kit (Ambion), and injected into embryos at the one- to two-cell stage.

In Vitro GST Pull-Down Assay

Wnt11r-FLAG-conditioned medium from transfected 293T cells was incubated with GST proteins and GST-UnpSV1ECD fusion proteins expressed in E. coli and absorbed to glutathione sepharose 4B, and, after washing, eluted proteins were detected with anti-FLAG antibody (1:1000, Sigma) and anti-GST antibody (1:5000, Sigma) on western blots as detailed in the Supplemental Data section.

Transient Transfection, Coimmunoprecipitation, and Western Blotting

Transient transfection and immunoprecipitation were carried out as previously described (Lu et al., 2004) with some modifications as detailed in the Supplemental Data section.

Transgenes

Transgenic lines were generated by microinjection of DNA as previously described (Thermes et al., 2002). The lines generated in this studies are Tg(hsp70l:unpSV1-myc)p1, Tg(hsp70l:unpFL-myc)p1, Tg(smyhc1:unpSV1-myc)p1, and Tg(smyhc1:unpFL-myc)p1 in accordance with ZFIN nomenclature.

Heat-Shock Condition

The embryos from the cross of $unplugged^{tbr307/tbr307}$; hsp70l:SV1(FL)-myc/+ to $unplugged^{tbr307/tbr307}$ were kept at 28°C to the desired stage before the heat shock. Each pair of embryos was then placed in 100 μl E3 medium in a single well of a 96-well PCR plate. Embryos were heat shocked at 38°C for 35 min at 2.5 hr intervals until they reached the appropriate stage. Transgenic embryos were identified from the control siblings by genotyping using the following primers: 5′TGACCAGATGCTCAAATCTGGTCTTTC3′ (forward) and 5′ATTAAGCTAGCGGTGAGGTCGCCCTA3′(reverse).

Live Imaging

Sixteen to twenty somite embryos were mounted in MatTek glass-bottom culture dishes using 1.2% NuSieve GTG agarose prepared in Ringers plus Tricane, and image stacks taken every 2 min using a Perkin Elmar UltraView spinning disk confocal equipped with a 63× lens. Growth cones were analyzed based on their morphologies during pathfinding.

Plasmid Construction

Standard molecular biology methods were used to generate unplugged FL, SV1, wnt11r, Dsh plasmids for protein expression, yeast two-hybrid and in situ hybridization as outlined in the Supplemental Data section.

In Situ Hybridization

Fluorescent in situ hybridizations were performed according to Downes et al. (2002) and Schneider and Granato (2006). Probes complementary to 5′UTR sequence of UnpSV1 (nt 1-340) or UnpFL unique coding sequence (nt 664–1012) were used. For wnt11r, probes complementary to wnt11r full-length sequences were used.

SUPPLEMENTAL DATA

The Supplemental Data include Supplemental Experimental Procedures, six figures, and two movies and can be found with this article online at http://www.neuron.org/supplemental/S0896-6273(09)00004-X.

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REFERENCES

Anderson, M.J., and Cohen, M.W. (1977). Nerve-induced and spontaneous redistribution of acetylcholine receptors on cultured muscle cells. J. Physiol. 268, 757–773.

Axelrod, J.D., Miller, J.R., Shulman, J.M., Moon, R.T., and Perrimon, N. (1998). Differential recruitment of Dishevelled provides signaling specificity in the planar cell polarity and Wingless signaling pathways. Genes Dev. 12, 2610–2622.

Bartscherer, K., and Boutros, M. (2008). Regulation of Wnt protein secretion and its role in gradient formation. EMBO Rep. 9, 977–982.

Becker, C.G., Schweitzer, J., Feldner, J., Becker, T., and Schachner, M. (2003). Tenascin-R as a repellent guidance molecule for developing optic axons in zebrafish. J. Neurosci. 23, 6232–6237.

Burden, S.J. (2000). Wnts as retrograde signals for axon and growth cone differentiation. Cell 100, 495–497.

Burden, S.J. (2002). Building the vertebrate neuromuscular synapse. J. Neurobiol. 53, 501–511.

Burden, S.J., Fuhrer, C., and Hubbard, S.R. (2003). Agrin/MuSK signaling: willing and Abl. Nat. Neurosci. 6, 653–654.

Cortes, F., Daggett, D., Bryson-Richardson, R.J., Neyt, C., Maule, J., Gautier, P., Hollway, G.E., Keenan, D., and Currie, P.D. (2003). Cadherin-mediated differential cell adhesion controls slow muscle cell migration in the developing zebrafish myotome. Dev. Cell 5, 865–876.

DeChiara, T.M., Bowen, D.C., Valenzuela, D.M., Simmons, M.V., Poueymirou, W.T., Thomas, S., Kinetz, E., Compton, D.L., Rojas, E., Park, J.S., et al. (1996). The receptor tyrosine kinase MuSK is required for neuromuscular junction formation in vivo. Cell 85, 501–512.

Downes, G.B., Waterbury, J.A., and Granato, M. (2002). Rapid in vivo labeling of identified zebrafish neurons. Genesis 34, 196–202.

Eisen, J.S., Myers, P.Z., and Westerfield, M. (1986). Pathway selection by growth-cones of identified motoneurons in live zebra fish embryos. Nature 320, 269–271.

Eisen, J.S., Pike, S.H., and Romancier, B. (1990). An identified motoneuron with variable fates in embryonic zebrafish. J. Neurosci. 10, 34–43.

Elworthy, S., Hargrave, M., Knight, R., Mebus, K., and Ingham, P.W. (2008). Expression of multiple slow myosin heavy chain genes reveals a diversity of zebrafish slow twitch muscle fibres with differing requirements for Hedgehog and Prdm1 activity. Development 135, 2115–2126.

Flanagan-Steet, H., Fox, M.A., Meyer, D., and Sanes, J.R. (2005). Neuromuscular synapses can form in vivo by incorporation of initially aneural postsynaptic specializations. Development 132, 4471–4481.

Frank, E., and Fischbach, G.D. (1979). Early events in neuromuscular junction formation in vitro: induction of acetylcholine receptor clusters in the postsynaptic membrane and morphology of newly formed synapses. J. Cell Biol. 83, 143–158.

Glass, D.J., Bowen, D.C., Stitt, T.N., Radziejewski, C., Bruno, J., Ryan, T.E., Gies, D.R., Shah, S., Mattsson, K., Burden, S.J., et al. (1996). Agrin acts via a MuSK receptor complex. Cell 85, 513–523.

Gros, J., Serralbo, O., and Marcelle, C. (2008). WNT11 acts as a directional cue to organize the elongation of early muscle fibres. Nature 457, 589–593.

Groves, J.A., Hammond, C.L., and Hughes, S.M. (2005). Fgf8 drives myogenic progression of a novel lateral fast muscle fibre population in zebrafish. Development 132, 4211–4222.

Hall, A.C., Lucas, F.R., and Salinas, P.C. (2000). Axonal remodeling and synaptic differentiation in the cerebellum is regulated by WNT-7a signaling. Cell 100, 525–535.

Halloran, M.C., Sato-Maeda, M., Warren, J.T., Su, F., Lele, Z., Krone, P.H., Kuwada, J.Y., and Shoji, W. (2000). Laser-induced gene expression in specific cells of transgenic zebrafish. Development 127, 1953–1960.

Heisenberg, C.P., Tada, M., Rauch, G.J., Saude, L., Concha, M.L., Geisler, R., Stemple, D.L., Smith, J.C., and Wilson, S.W. (2000). Silberblick/Wnt11 mediates convergent extension movements during zebrafish gastrulation. Nature 405, 76–81.

Henriquez, J.P., Webb, A., Bence, M., Bildsoe, H., Sahores, M., Hughes, S.M., and Salinas, P.C. (2008). Wnt signaling promotes AChR aggregation at the neuromuscular synapse in collaboration with agrin. Proc. Natl. Acad. Sci. USA 105, 18812–18817.

Hikasa, H., Shibata, M., Hiratani, I., and Taira, M. (2002). The Xenopus receptor tyrosine kinase Xror2 modulates morphogenetic movements of the axial mesoderm and neuroectoderm via Wnt signaling. Development 129, 5227–5239.

Jones, G., Moore, C., Hashemolhosseini, S., and Brenner, H.R. (1999). Constitutively active MuSK is clustered in the absence of agrin and induces ectopic postsynaptic-like membranes in skeletal muscle fibers. J. Neurosci. 19, 3376–3383.

Kim, N., and Burden, S.J. (2008). MuSK controls where motor axons grow and form synapses. Nat. Neurosci. 11, 19–27.

Kim, C.H., Xiong, W.C., and Mei, L. (2003). Regulation of MuSK expression by a novel signaling pathway. J. Biol. Chem. 278, 38522–38527.

Kim, N., Stiegler, A.L., Cameron, T.O., Hallock, P.T., Gomez, A.M., Huang, J.H., Hubbard, S.R., Dustin, M.L., and Burden, S.J. (2008). Lrp4 is a receptor for Agrin and forms a complex with MuSK. Cell 135, 334–342.

Klassen, M.P., and Shen, K. (2007). Wnt signaling positions neuromuscular connectivity by inhibiting synapse formation in C. elegans. Cell 130, 704–716.

Kummer, T.T., Misgeld, T., and Sanes, J.R. (2006). Assembly of the postsynaptic membrane at the neuromuscular junction: paradigm lost. Curr. Opin. Neurobiol. 16, 74–82.

Lefebvre, J.L., Ono, F., Puglielli, C., Seidner, G., Franzini-Armstrong, C., Brehm, P., and Granato, M. (2004). Increased neuromuscular activity causes axonal defects and muscular degeneration. Development 131, 2605–2618.

Lefebvre, J.L., Jing, L., Becaficco, S., Franzini-Armstrong, C., and Granato, M. (2007). Differential requirement for MuSK and dystroglycan in generating patterns of neuromuscular innervation. Proc. Natl. Acad. Sci. USA 104, 2483–2488.

Lin, W., Burgess, R.W., Dominguez, B., Pfaff, S.L., Sanes, J.R., and Lee, K.F. (2001). Distinct roles of nerve and muscle in postsynaptic differentiation of the neuromuscular synapse. Nature 410, 1057–1064.

Lin, W., Dominguez, B., Yang, J., Aryal, P., Brandon, E.P., Gage, F.H., and Lee, K.F. (2005). Neurotransmitter acetylcholine negatively regulates neuromuscular synapse formation by a Cdk5-dependent mechanism. Neuron 46, 569–579.

Lin, S., Landmann, L., Ruegg, M.A., and Brenner, H.R. (2008). The role of nerve- versus muscle-derived factors in mammalian neuromuscular junction formation. J. Neurosci. 28, 3333–3340.

Liu, D.W.C., and Westerfield, M. (1992). Clustering of muscle acetylcholine receptors requires motoneurons in live embryos, but not in cell culture. J. Neurosci. 12, 1859–1866.

Lu, W., Yamamoto, V., Ortega, B., and Baltimore, D. (2004). Mammalian Ryk is a Wnt coreceptor required for stimulation of neurite outgrowth. Cell 119, 97–108.

Luo, Z.G., Wang, Q., Zhou, J.Z., Wang, J., Luo, Z., Liu, M., He, X., Wynshaw-Boris, A., Xiong, W.C., Lu, B., and Mei, L. (2002). Regulation of AChR clustering by Dishevelled interacting with MuSK and PAK1. Neuron 35, 489–505.

Masuda, T., Fukamauchi, F., Takeda, Y., Fujisawa, H., Watanabe, K., Okado, N., and Shiga, T. (2004). Developmental regulation of notochord-derived repulsion for dorsal root ganglion axons. Mol. Cell. Neurosci. 25, 217–227.

Matsui, T., Raya, A., Kawakami, Y., Callol-Massot, C., Capdevila, J., Rodriguez-Esteban, C., and Izpisua Belmonte, J.C. (2005). Noncanonical Wnt signaling regulates midline convergence of organ primordia during zebrafish development. Genes Dev. 19, 164–175.

Misgeld, T., Kummer, T.T., Lichtman, J.W., and Sanes, J.R. (2005). Agrin promotes synaptic differentiation by counteracting an inhibitory effect of neurotransmitter. Proc. Natl. Acad. Sci. USA 102, 11088–11093.

Moore, C., Leu, M., Muller, U., and Brenner, H.R. (2001). Induction of multiple signaling loops by MuSK during neuromuscular synapse formation. Proc. Natl. Acad. Sci. USA 98, 14655–14660.

Oishi, I., Suzuki, H., Onishi, N., Takada, R., Kani, S., Ohkawara, B., Koshida, I., Suzuki, K., Yamada, G., Schwabe, G.C., et al. (2003). The receptor tyrosine kinase Ror2 is involved in non-canonical Wnt5a/JNK signalling pathway. Genes Cells 8, 645–654.

Okada, K., Inoue, A., Okada, M., Murata, Y., Kakuta, S., Jigami, T., Kubo, S., Shiraishi, H., Eguchi, K., Motomura, M., et al. (2006). The muscle protein Dok-7 is essential for neuromuscular synaptogenesis. Science 312, 1802–1805.

Packard, M., Koo, E.S., Gorczyca, M., Sharpe, J., Cumberledge, S., and Budnik, V. (2002). The Drosophila Wnt, wingless, provides an essential signal for pre- and postsynaptic differentiation. Cell 111, 319–330.

Panzer, J.A., Gibbs, S.M., Dosch, R., Wagner, D., Mullins, M.C., Granato, M., and Balice-Gordon, R.J. (2005). Neuromuscular synaptogenesis in wild-type and mutant zebrafish. Dev. Biol. 285, 340–357.

Panzer, J.A., Song, Y., and Balice-Gordon, R.J. (2006). In vivo imaging of preferential motor axon outgrowth to and synaptogenesis at prepatterned acetylcholine receptor clusters in embryonic zebrafish skeletal muscle. J. Neurosci. 26, 934–947.

Rauch, G.J., Hammerschmidt, M., Blader, P., Schauerte, H.E., Strahle, U., Ingham, P.W., McMahon, A.P., and Haffter, P. (1997). Wnt5 is required for tail formation in the zebrafish embryo. Cold Spring Harb. Symp. Quant. Biol. 62, 227–234.

Sanes, J.R., and Lichtman, J.W. (2001). Induction, assembly, maturation and maintenance of a postsynaptic apparatus. Nat. Rev. Neurosci. 2, 791–805.

Schneider, V.A., and Granato, M. (2006). The myotomal diwanka (lh3) glycosyltransferase and type XVIII collagen are critical for motor growth cone migration. Neuron 50, 683–695.

Schweitzer, J., Becker, T., Lefebvre, J., Granato, M., Schachner, M., and Becker, C.G. (2005). Tenascin-C is involved in motor axon outgrowth in the trunk of developing zebrafish. Dev. Dyn. 234, 550–566.

Speese, S.D., and Budnik, V. (2007). Wnts: up-and-coming at the synapse. Trends Neurosci. 30, 268–275.

Strigini, M., and Cohen, S.M. (2000). Wingless gradient formation in the Drosophila wing. Curr. Biol. 10, 293–300.

Thermes, V., Grabher, C., Ristoratore, F., Bourrat, F., Choulika, A., Wittbrodt, J., and Joly, J.S. (2002). I-SceI meganuclease mediates highly efficient transgenesis in fish. Mech. Dev. 118, 91–98.

Vock, V.M., Ponomareva, O.N., and Rimer, M. (2008). Evidence for muscle-dependent neuromuscular synaptic site determination in mammals. J. Neurosci. 28, 3123–3130.

Wallingford, J.B., Rowning, B.A., Vogeli, K.M., Rothbacher, U., Fraser, S.E., and Harland, R.M. (2000). Dishevelled controls cell polarity during Xenopus gastrulation. Nature 405, 81–85.

Wang, J., Jing, Z., Zhang, L., Zhou, G., Braun, J., Yao, Y., and Wang, Z.Z. (2003). Regulation of acetylcholine receptor clustering by the tumor suppressor APC. Nat. Neurosci. 6, 1017–1018.

Weatherbee, S.D., Anderson, K.V., and Niswander, L.A. (2006). LDL-receptor-related protein 4 is crucial for formation of the neuromuscular junction. Development 133, 4993–5000.

Wodarz, A., and Nusse, R. (1998). Mechanisms of Wnt signaling in development. Annu. Rev. Cell Dev. Biol. 14, 59–88.

Xu, Y.K., and Nusse, R. (1998). The Frizzled CRD domain is conserved in diverse proteins including several receptor tyrosine kinases. Curr. Biol. 8, R405–R406.

Yang, X., Arber, S., William, C., Li, L., Tanabe, Y., Jessell, T.M., Birchmeier, C., and Burden, S.J. (2001). Patterning of muscle acetylcholine receptor gene expression in the absence of motor innervation. Neuron 30, 399–410.

Zecca, M., Basler, K., and Struhl, G. (1996). Direct and long-range action of a wingless morphogen gradient. Cell 87, 833–844.

Zeller, J., Schneider, V., Malayaman, S., Higashijima, S., Okamoto, H., Gui, J., Lin, S., and Granato, M. (2002). Migration of zebrafish spinal motor nerves into the periphery requires multiple myotome-derived cues. Dev. Biol. 252, 241–256.

Zhang, J., and Granato, M. (2000). The zebrafish unplugged gene controls motor axon pathway selection. Development 127, 2099–2111.

Zhang, J., Lefebvre, J.L., Zhao, S., and Granato, M. (2004). Zebrafish unplugged reveals a role for muscle-specific kinase homologs in axonal pathway choice. Nat. Neurosci. 7, 1303–1309.

Zhang, B., Luo, S., Dong, X.P., Zhang, X., Liu, C., Luo, Z., Xiong, W.C., and Mei, L. (2007). Beta-catenin regulates acetylcholine receptor clustering in muscle cells through interaction with rapsyn. J. Neurosci. 27, 3968–3973.

Zhang, B., Luo, S., Wang, Q., Suzuki, T., Xiong, W.C., and Mei, L. (2008). LRP4 serves as a coreceptor of Agrin. Neuron 60, 285–297.

Zhou, H., Glass, D.J., Yancopoulos, G.D., and Sanes, J.R. (1999). Distinct domains of MuSK mediate its abilities to induce and to associate with postsynaptic specializations. J. Cell Biol. 146, 1133–1146.