

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

Lili Jing,¹ Julie L. Lefebvre,^{1,2} Laura R. Gordon,¹ and Michael Granato^{1,*}

¹Department of Cell and Developmental Biology, University of Pennsylvania School of Medicine, Philadelphia, PA 19104-6058, USA

²Present address: Harvard University, Cambridge, MA 02138, USA

*Correspondence: granatom@mail.med.upenn.edu

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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 pre patterning 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 pre patterning (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

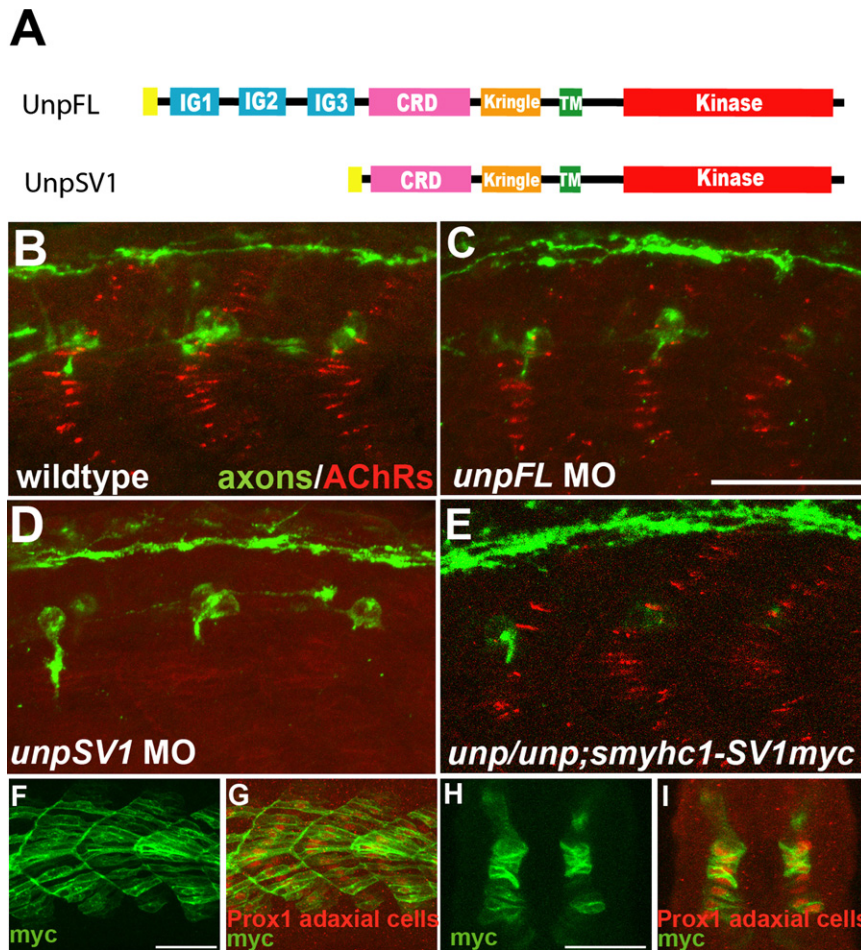


Figure 1. Unplugged Controls AChR Pre-patterning

(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) Unplugged FL MO injection does not affect AChR pre-patterning. (D) Unplugged SV1 MO injection causes complete absence of AChR pre-patterning. (E) Unplugged SV1 expression in adaxial cells restores AChR pre-patterning in *unplugged* mutant embryos.

(F and G) Lateral views and cross-sectional views (H and I) of 17 hpf *Tg(smyhc1:UnpluggedSV1myc)* embryos stained with anti-myc (green) and anti-Prox1 (red), which labels the nuclei of adaxial cells. Scale bars, 50 μ m.

the expression patterns of *unplugged* FL and SV1 using isoform-specific probes. Before and during the time of AChR pre-patterning, *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 pre-patterning.

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 pre-patterning 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 pre-patterning, consistent with their differential expression patterns (Figures 1B–1D). To confirm that the *unplugged* SV1 receptor is indeed responsible for AChR pre-patterning, 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 pre-patterning (Figure 1E). Thus, similar to its requirement in axonal pathfinding, the *unplugged* SV1 receptor, which lacks the Ig domains, is responsible for AChR pre-patterning, consistent with the idea that in vivo both processes share a common signaling mechanism.

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 pre-patterned 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 pre-patterning.

We have previously shown that *unplugged* null mutants lack all AChR pre-patterning 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 pre-patterning in adaxial fibers, we first analyzed

ulin-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 pre-patterning 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 pre-patterning, consistent with their differential expression patterns (Figures 1B–1D). To confirm that the *unplugged* SV1 receptor is indeed responsible for AChR pre-patterning, 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 pre-patterning (Figure 1E). Thus, similar to its requirement in axonal pathfinding, the *unplugged* SV1 receptor, which lacks the Ig domains, is responsible for AChR pre-patterning, consistent with the idea that in vivo both processes share a common signaling mechanism.

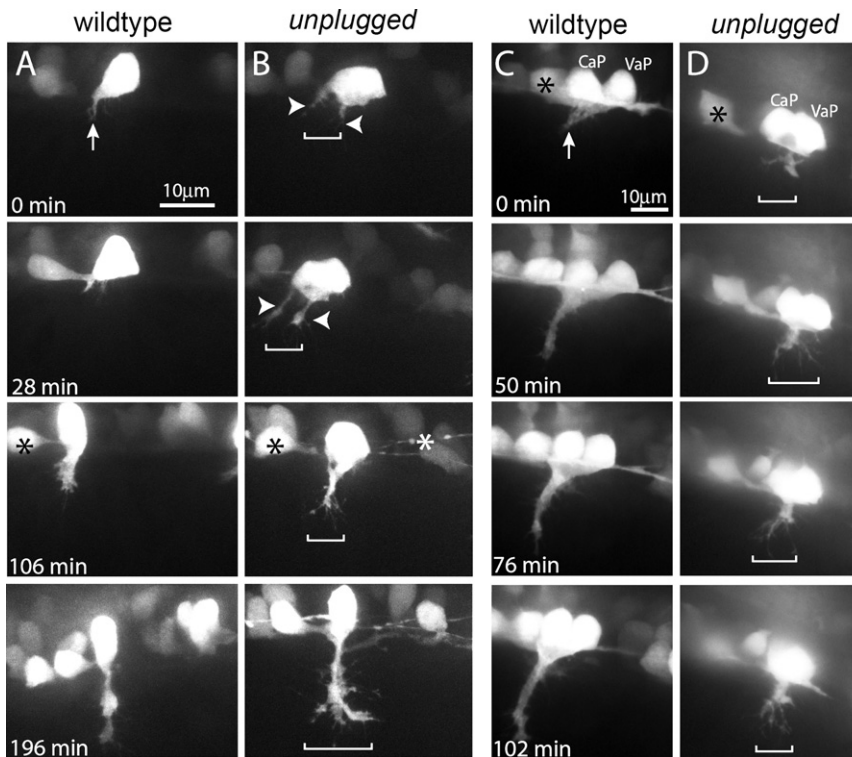


Figure 2. *unplugged* Restricts Navigating Growth Cones to a Central Muscle Zone

(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]). Similarly, 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.

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

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

We then asked if *unplugged*/MuSK co-ordinates axonal pathfinding and AChR pre patterning. While we had previously shown that in the zebrafish embryo *unplugged*/MuSK is critical for axonal guidance after the period of AChR pre patterning (Zhang and Granato, 2000), we decided to examine axonal pathfinding at an earlier stage during the period of AChR pre patterning. 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 filopodia, 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

The Noncanonical *wnt11r* Ligand Plays a Role in AChR Pre patterning 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*^{p31^{CD}} 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).

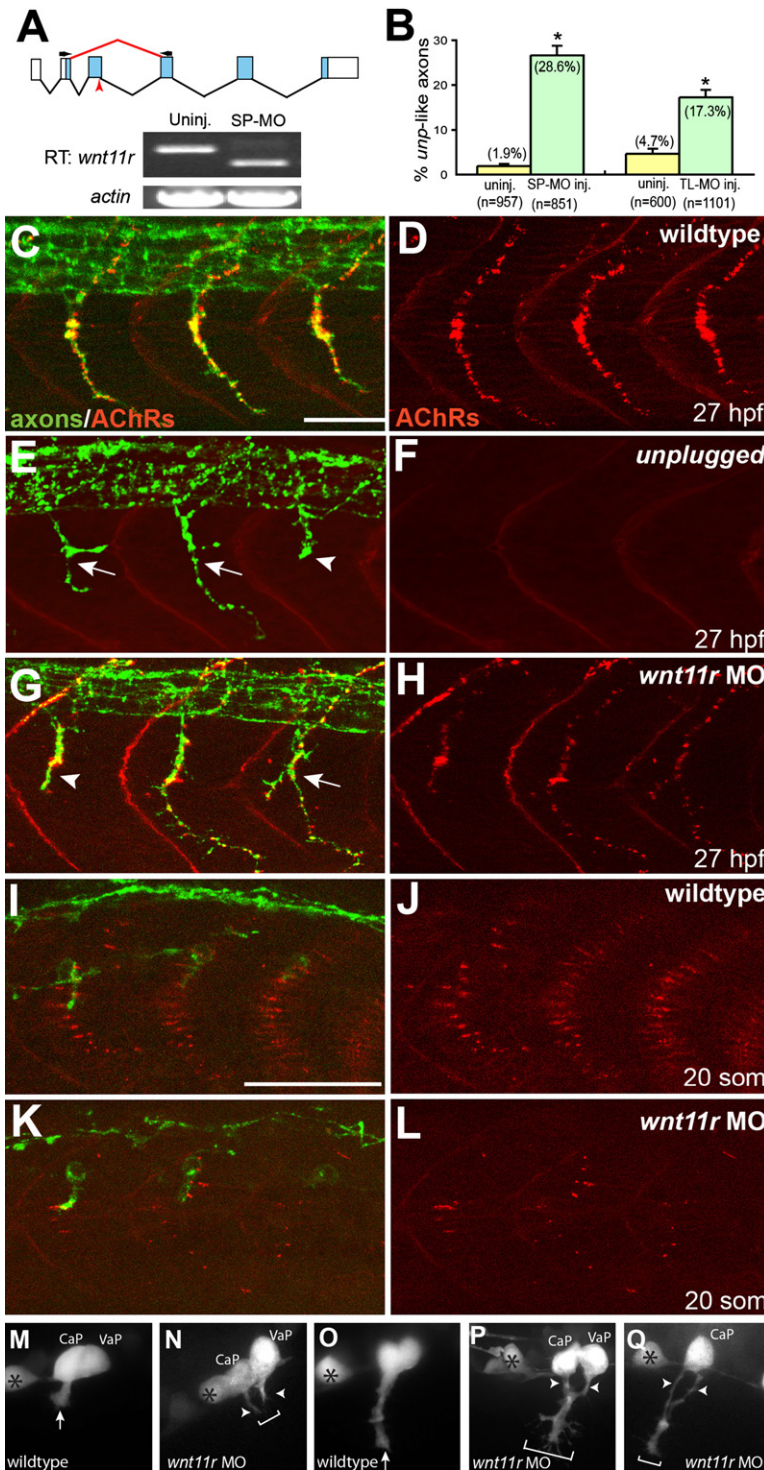


Figure 3. *wnt11r* Is Critical for Axonal Guidance and AChR Prepatterning

(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 morpholino. 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 prepattern (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 μ m.

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

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 prepatterning 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 filopodia (Figure 3M–Q). Thus, *wnt11r* morphants display AChR prepatterning 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 Δ CRD-myc) were cotrans-

ected 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 SV1 Δ CRD, 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 prepatterning. Injection of mRNA encoding for *wnt11r* or *unplugged* SV1 into wild-type embryos revealed no difference in AChR prepatterning (Figures 4E, 4F, and 4I). In contrast, coinjection of both *wnt11r* and *unplugged* SV1 mRNAs induced ectopic AChR clusters (Figures 4G and 4I). To test whether the *unplugged* CRD is required in vivo for *wnt11r*-induced AChR prepatterning, we also coinjected *wnt11r* and *unplugged* SV1 Δ CRD (lacking the Wnt-binding domain), which did not increase AChR prepatterning (Figures 4H and 4I). Thus, our results show that *unplugged*/MuSK and *wnt11r* are both mutually required for induction of the AChR prepatterning, consistent with a ligand-dependent mode of action.

Blocking *dishevelled* 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* promoter 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

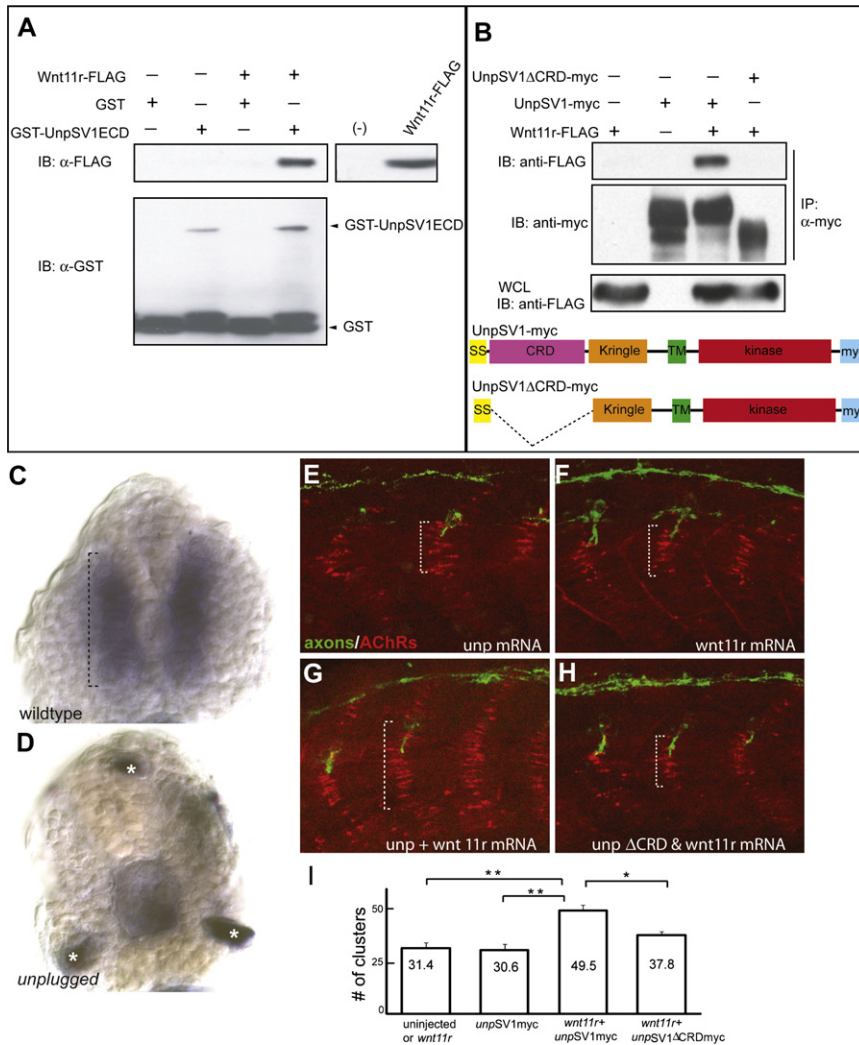


Figure 4. Wnt11r Binds to UnpSV1 and Overexpressions of wnt11r and unpSV1 Increase Pre patterning

(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 pre patterning (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 pre patterned 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 Δ 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 pre pattern 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 pre patterning.

Synapses Form in the Absence of AChR Pre pattern

Our results show that *unplugged*/MuSK and *wnt11r* play critical roles in initiating the AChRs pre pattern. These pre patterned AChR clusters can be incorporated into neuromuscular junctions, but is AChR pre pattern essential for synapse formation? To answer this question, we generated multiple inducible *unplugged*/MuSK transgenic lines, in which the heat-shock protein 70 (*hsp70*) 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(*hsp70*:*unplugged* FL-myc); *unplugged*^(br307/br307) or Tg(*hsp70*:*unplugged* SV1-myc); *unplugged*^(br307/br307) embryos lacked all AChR pre pattern and neuromuscular synapses (Figures S6A–S6D).

We then used continuous heat-shock treatment to induce expression of Tg(*hsp70*:*unplugged* FL-myc) or Tg(*hsp70*:*unplugged* SV1-myc) in *unplugged*^(br307/br307) embryos, starting several hours before the first AChR clusters become detectable.

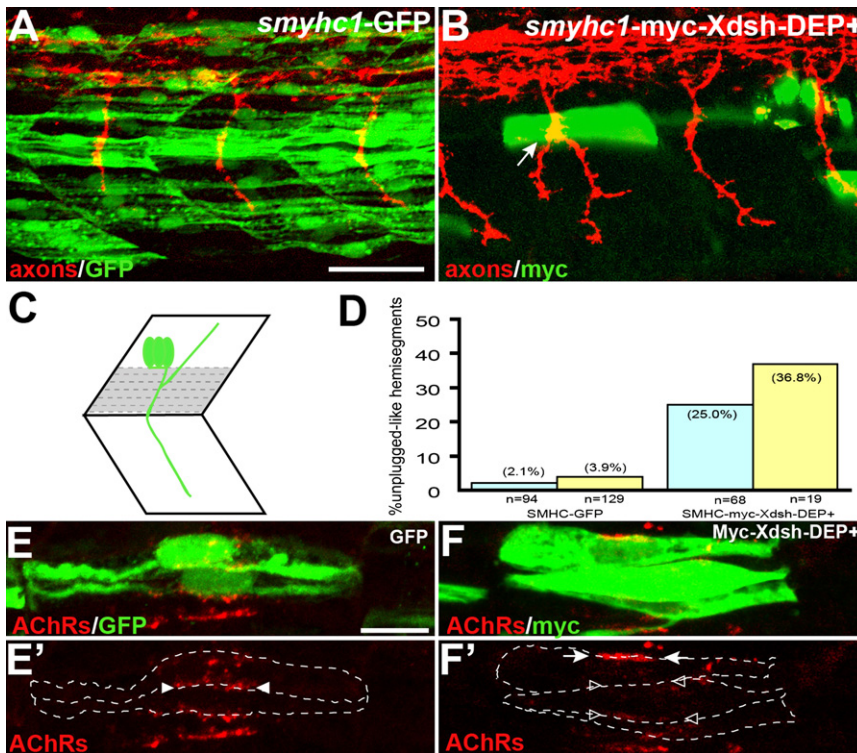


Figure 5. Inhibition of the Noncanonical Dsh Pathway in Adaxial Fibers

(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 μ m, (E) 10 μ m.

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 *unplugged*FL under the control of the adaxial-specific promoter Tg(*smyhc1*:*unplugged*FL-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 pre-patterning, 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

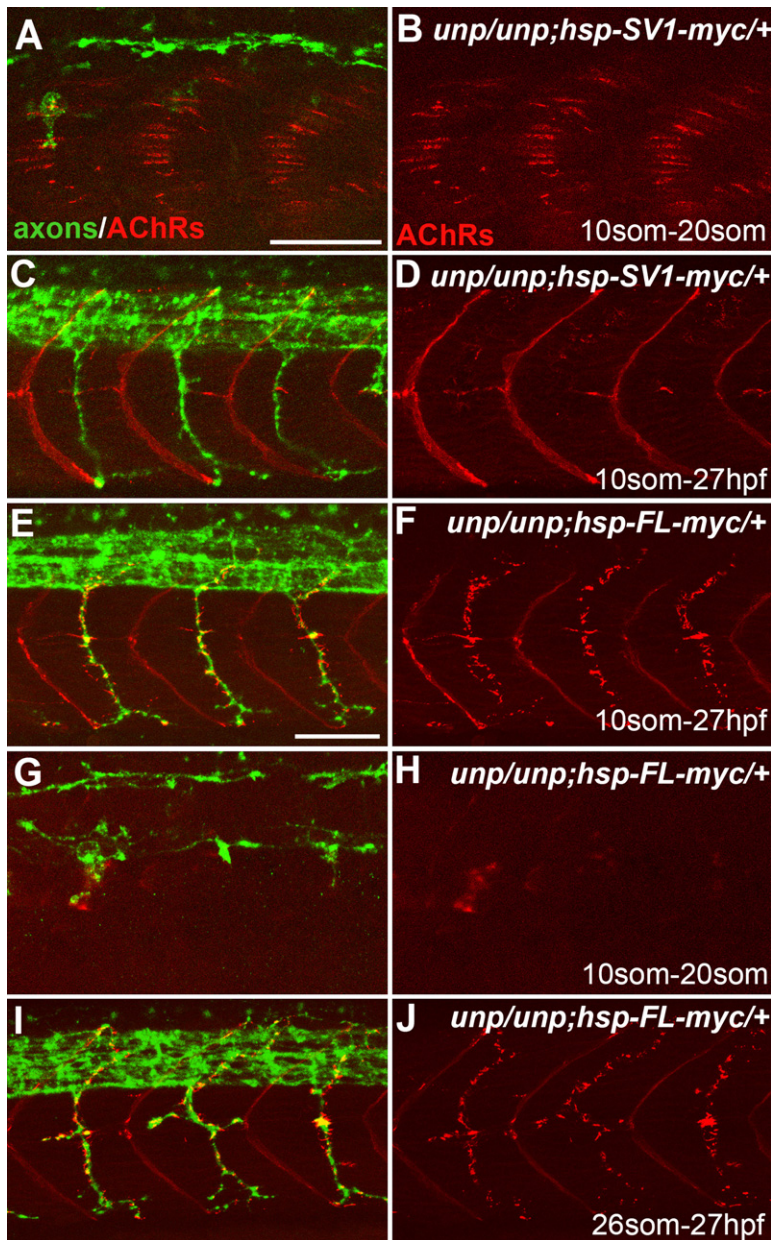


Figure 6. Neuromuscular Synapses Form in the Absence of AChR Prepattern

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 pre-patterning, was sufficient to rescue neuromuscular synapses.

Scale bars, 50 μ m.

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 pre-patterning 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 *dishevelled*-dependent noncanonical Wnt pathway in adaxial cells also causes defects in AChR pre-patterning and axonal pathfinding.

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).

Together, these data suggest that in response to Wnt ligands muscle cells enable an *unplugged*/MuSK signaling cascade that restricts growth cones and AChR prepatterning 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 prepatterning 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 dorso-lateral 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 prepatterning 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 prepatterning 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 prepatterning are confined.

AChR Prepatterning Is Dispensable for NMJ Formation

In zebrafish, prepatterning 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 prepatterning 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 prepatterning 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 “myo-centric” 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 prepatterning, suggesting that such prepatterning 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 prepatterning 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 prepatterning 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 post-synaptic processes to a common, narrow domain. Intriguingly,

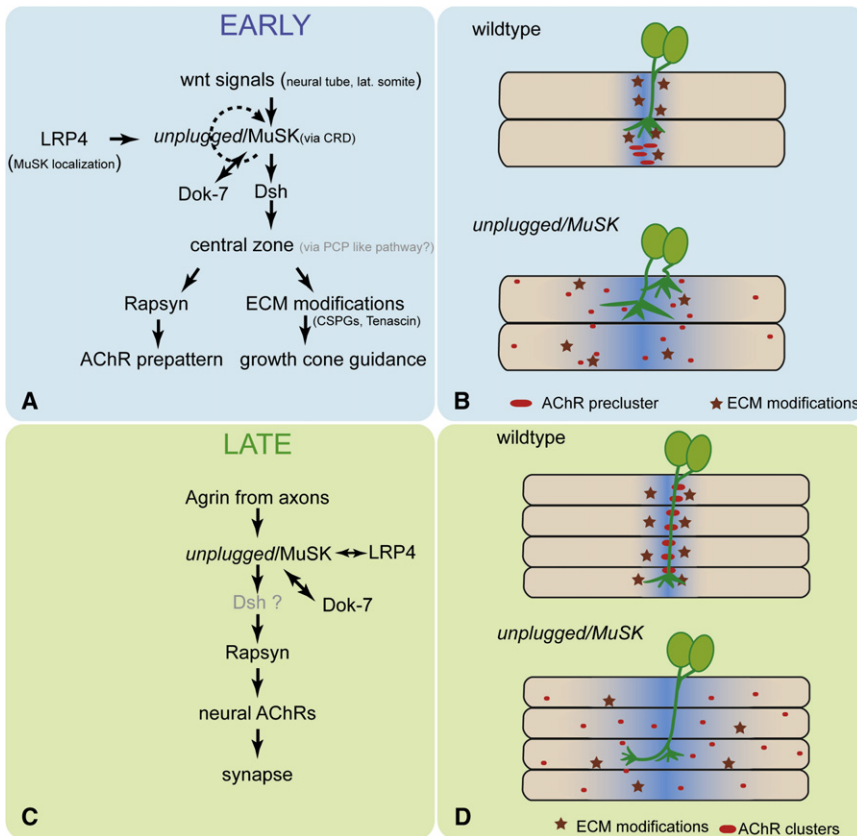


Figure 7. *unplugged*/MuSK Signaling during Synapse Formation

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 clusters 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 pre patterning (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 collagenase (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 α BTX (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'TTTTCTCAGTAACTCACCTCGTTC3') 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'-TCCTCACATTCTGCTGCTGTC-3' (forward) and 5'-TCTTCATCTT CATTGGGCATC-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 μ l 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 Elmer UltraView spinning disk confocal equipped with a 63 \times 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](http://www.neuron.org/supplemental/S0896-6273(09)00004-X).

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