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Mechanisms of Unplugged/Musk-Mediated Neuromuscular Synapse Formation

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Mechanisms of Unplugged/Musk-Mediated Neuromuscular Synapse Formation

Abstract
Formation of neuromuscular connectivity involves proper navigation of motor axons from the spinal cord to their muscle targets as well as precise alignment of these motor axons with acetylcholine receptors (AChRs) on the muscle surface. One key molecular player in this process is the muscle specific kinase, unplugged/MuSK, which is expressed only in muscle cells. When activated by the nerve-derived ligand Agrin, Unplugged/MuSK triggers the accumulation of AChR clusters in the center of muscle fibers. Even before the arrival of motor axons, AChRs accumulate in the center of muscle fibers in an unplugged/MuSK-dependent manner and prefigure the sites of the first neuromuscular synapses. However, how Unplugged/MuSK activation translates into centralized AChR clustering is not well understood. The lab previously showed that Wnt11r acts through Unplugged/MuSK to restrict anueral AChR clusters and motor axons to the center of the muscle fiber. Despite a high efficiency of knockdown, the \textit{wnt11r} knockdown phenotype was incompletely penetrant when compared to \textit{unplugged} null mutants, which suggested that other Wnts might be contributing to unplugged/MuSK-mediated signaling. In this thesis work, I show that loss of two noncanonical Wnts, \textit{wnt11r} and \textit{wnt4a}, results in axon guidance errors as well as a complete loss of anueral AChR clusters, identical to the loss of the unplugged/MuSK receptor. In vivo, Wnt11r/4a initiate Unplugged/MuSK translocation from the muscle membrane to recycling endosomes, and this transition is critical for AChR localization to future synaptic sites. Moreover, I show that components of the planar cell polarity pathway coloclate to recycling endosomes, and that this localization is unplugged/MuSK dependent. Knockdown of several core components of the planar cell polarity pathway disrupts Unplugged/MuSK translocation to endosomes, AChR localization and axonal guidance. I propose that Wnt-induced trafficking of the Unplugged/MuSK receptor to endosomes initiates a signaling cascade to align pre- with postsynaptic elements. Collectively, these findings suggest a general mechanism by which Wnt signals shape synaptic connectivity through localized receptor endocytosis.

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MECHANISMS OF UNPLUGGED/MUSK-MEDIATED
NEUROMUSCULAR SYNAPSE FORMATION

Laura Rae Gordon
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in
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Presented to the Faculties of the University of Pennsylvania
in
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Degree of Doctor of Philosophy
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ABSTRACT

MECHANISMS OF UNPLUGGED/MUSK-MEDIATED NEUROMUSCULAR SYNAPSE FORMATION

Laura Rae Gordon
Michael Granato

Formation of neuromuscular connectivity involves proper navigation of motor axons from the spinal cord to their muscle targets as well as precise alignment of these motor axons with acetylcholine receptors (AChRs) on the muscle surface. One key molecular player in this process is the muscle specific kinase, unplugged/MuSK, which is expressed only in muscle cells. When activated by the nerve-derived ligand Agrin, Unplugged/MuSK triggers the accumulation of AChR clusters in the center of muscle fibers. Even before the arrival of motor axons, AChRs accumulate in the center of muscle fibers in an unplugged/MuSK-dependent manner and prefigure the sites of the first neuromuscular synapses. However, how Unplugged/MuSK activation translates into centralized AChR clustering is not well understood. The lab previously showed that Wnt11r acts through Unplugged/MuSK to restrict anueral AChR clusters and motor axons to the center of the muscle fiber. Despite a high efficiency of knockdown, the wnt11r knockdown phenotype was incompletely penetrant when compared to unplugged null mutants, which suggested that other Wnts might be contributing to unplugged/MuSK-mediated signaling. In this thesis work, I show that loss of two noncanonical Wnts, wnt11r and wnt4a, results in axon guidance errors as well as a complete loss of anueral AChR clusters, identical to
the loss of the unplugged/MuSK receptor. In vivo, Wnt11r/4a initiate Unplugged/MuSK translocation from the muscle membrane to recycling endosomes, and this transition is critical for AChR localization to future synaptic sites. Moreover, I show that components of the planar cell polarity pathway colocalize to recycling endosomes, and that this localization is unplugged/MuSK dependent. Knockdown of several core components of the planar cell polarity pathway disrupts Unplugged/MuSK translocation to endosomes, AChR localization and axonal guidance. I propose that Wnt-induced trafficking of the Unplugged/MuSK receptor to endosomes initiates a signaling cascade to align pre- with postsynaptic elements. Collectively, these findings suggest a general mechanism by which Wnt signals shape synaptic connectivity through localized receptor endocytosis.
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CHAPTER 1

INTRODUCTION

Neuromuscular Junction Development

Structure of the Neuromuscular Junction

Vertebrate motor neuron cell bodies are located in the spinal cord. During development, motor neuron axons exit the spinal cord at predetermined sites and continue extending into the periphery. Once in the periphery, motor axons navigate to their proper muscle targets and make synaptic contacts. This innervation primarily occurs at a single, focal site in the center of the muscle fiber with one motor neuron innervating multiple muscle fibers.

Nerve excitation triggers presynaptic release of acetylcholine (ACh), which is contained in vesicles that are enriched at active zones, or sites of transmitter release, in the presynaptic terminal. ACh then traverses the ~50nm synaptic cleft and binds to nicotinic acetylcholine receptors (AChRs) presented on the crests of membranous folds on the surface of the muscle fiber. AChRs are typically localized to the peaks of these folds as well as partially down the sides while calcium channels are concentrated in the troughs (reviewed in Sanes and Lichtman, 2001). This precise alignment of pre- and postsynaptic elements allows for quick and efficient chemical transmission.

The architecture of the neuromuscular junction develops over time, spanning a period of nearly three weeks in mice. After the initial contact between the nerve and muscle is established, the number of AChRs increases, the shape of the synapse is refined and multiple sites of innervation are reduced to a single site (reviewed in Sanes and Lichtman, 2001). In mice, embryonic AChR subunits are replaced by adult subunits (Witzemann, 2006), but this developmental transition of the complement of AChR
subunits does not occur in zebrafish (Etard et al., 2005). At the mature neuromuscular junction, the number of AChRs at the synaptic site, which can range from 10,000-20,000 AChRs/um$^2$, remains constant, reflecting a balance of receptor insertion and degradation (reviewed in St John, 2009).

**Zebrafish Myotome Development**

Developing somites are composed primarily of myotomal tissue that consists of two spatially segregated subtypes, fast and slow-twitch muscle fibers (reviewed in Stickney et al., 2000). In zebrafish, the myoseptal pathway along which motor neuron axons extend is initially paved by a single layer of slow-twitch adaxial cells. While some of these adaxial cells migrate laterally and are replaced by fast-twitch muscle precursors (Cortes et al., 2003; Devoto et al., 1996), 2-6 cells per somite known as the muscle pioneers, remain at the choice point and are contacted directly by growth cones (Felsenfeld et al., 1991; Melançon et al., 1997).

Although slow-twitch adaxial fibers contain a single, centrally-positioned nucleus (Roy et al., 2001), most vertebrate skeletal muscle fibers, including zebrafish fast-twitch muscles, are multinucleated and may contain hundreds to thousands of synaptic nuclei that are clustered directly beneath the synapse. The enrichment of synaptic nuclei in polynucleated fibers primarily arises from multiple symmetrical fusions of precursor myoblasts on either side of the synapse during early embryonic development (Roy et al., 2001).

Synaptic nuclei are enriched in the synaptic anchoring protein Syne-1 (Zhang et al., 2007b) and preferentially transcribe a number of genes involved in synaptic maintenance and function such as AChR, Neuregulin (NRG1) and Utrophin (Gramolini et al., 1999; Jo et al., 1995; Klarsfeld et al., 1991; Sanes et al., 1991; Simon et al., 1992). The selective transcription of AChRs at synaptic nuclei has been proposed to result in a
central enrichment of AChRs in the center of the fiber (Apel et al., 1997; Gautam et al., 1995). However, this view was challenged by the finding that disrupting the central localization of synaptic nuclei by mutating syne-1 in mice did not perturb central AChR clustering (Zhang et al., 2007a). Additionally, it is not known how central clustering of AChRs is achieved in zebrafish slow-twitch muscle fibers, which have a single nucleus. Understanding the mechanism driving the initial localization and clustering of AChRs in the center of zebrafish mononucleated slow-twitch muscle fibers is a focus of this thesis work.

**Zebrafish Neuromuscular Junction Formation**

The zebrafish peripheral nervous system initially contains only three primary motor neurons per somitic hemisegment (Beattie, 2000; Eisen et al., 1986). The primary motor neurons, caudal primary (CaP), middle primary (MiP) and rostral primary (RoP), are named for the relative positions of their cell bodies in the spinal cord. In addition to differing in position, the motor neurons can be distinguished by their combinatorial expression of LIM homeodomain factors. RoP and MiP express *lim3* and *islet 1* while CaP expresses *lim3* and *islet 2* (Appel et al., 1995). A fourth motor neuron subtype, variable primary (VaP), accompanies CaP in roughly half of somitic hemisegments, but disappears by 32 hours post fertilization (hpf) (Eisen et al., 1990).

Upon exiting the spinal cord, all of the motor neuron axons migrate ventrally along the medial surface of the somite, perpendicular to the striated muscle fibers, and continue to follow this common path until they reach the choice point at the horizontal myoseptum. At the choice point, the growth cones pause and then forge highly stereotyped, independent trajectories into the periphery where they make *en passant* contacts with target muscle fibers (Eisen et al., 1986; Myers et al., 1986; Westerfield et al., 1986). Starting around 26hpf, a second wave of motor neurons, known as
secondary motor neurons, follow the path forged by the pioneering primary motor neurons. Studies in which primary motor neurons are ablated show that the second wave of migration is independent of the primary migration (Pike et al., 1992).

The formation of functional synapses between motor neurons and muscle cells can be broken into two distinct phases; an early phase and a late phase. The early phase, which is the focus of this thesis work, involves the pre-clustering of AChRs in the center of the muscle fiber prior to the arrival of the nerve. The late phase is characterized by the arrival of the motor neuron at its muscle target and the stabilization of appropriate synapses. Stabilization of synapses during this late phase is coordinated by the secreted, nerve-derived protein, Agrin.

Agrin, a 200kD heparin sulfate proteoglycan, was first isolated from *Torpedo californica* for its ability to induce post-translational AChR clustering in cultured myotubes (Godfrey, 1984). Agrin is secreted by muscles, Schwann cells and nerves, but the nerve-derived form, also known as z⁺ Agrin, is better able to induce AChR clustering than z⁻ Agrin (Gesemann et al., 1995; Hoch et al., 1994). Agrin does not appear to bind directly to the extracellular domain of MuSK. Rather, Agrin is thought to interact indirectly through Lrp4, which forms a complex with MuSK (Kim et al., 2008b). Agrin induces the rapid tyrosine phosphorylation of MuSK at a juxtamembrane tyrosine (Y553) (Glass et al., 1996) followed by MuSK internalization (Zhu et al., 2008) as well as AChR aggregation (DeChiara et al., 1996). Consequently, Agrin mutants exhibit a dramatic loss of synaptic AChR clusters (Gautam et al., 1996). Although Agrin is required for the late phase of synaptic development, it is not involved in the earlier phase, as loss of Agrin does not affect AChR prepatterning (Lin et al., 2001; Yang et al., 2001).
Embryos completely lacking all motor nerves have AChR clusters that persist longer than those in Agrin mutants (Lin et al., 2001), suggesting that the nerve provides a dispersal cue for AChRs. This negative signal was identified as ACh, which upon release by the nerve terminal, has the opposite effect of Agrin and scatters inappropriate AChR clusters (Lin et al., 2005). Therefore, the precise clustering of AChRs directly beneath the nerve terminal is a product of both positive clustering cues (Agrin) and negative dispersal cues (ACh). While this late phase of neuromuscular formation is fairly well characterized, the early stage is still not well understood.

The early phase, which occurs prior to the arrival of the motor nerve, is characterized by the loose clustering of AChRs along the center of the muscle membrane precisely where the motor nerve will eventually crawl. Many of these prepatterned AChRs are ultimately incorporated into mature synapses, but they are not required for motor axon outgrowth (Panzer et al., 2006). Just as prepatterned clusters are not required for axon outgrowth, the existence of these clusters does not depend on the presence of the nerve or the nerve-derived ligand Agrin (Lin et al., 2001). Formation of prepatterned AChR clusters is, however, dependent on the receptor tyrosine kinase MuSK (Bowen et al., 1998; Lin et al., 2001; Yang et al., 2001).

*Unplugged*/MuSK Structure and Function

The *unplugged* mutant was first identified in a large-scale genetic screen for its reduced embryonic locomotion phenotype (Granato et al., 1996), and positional cloning revealed that *unplugged* encodes a homolog of the mammalian muscle-specific kinase (MuSK) (Zhang et al., 2004). The two homologues each contain three immunoglobulin-like (Ig) domains, a frizzled-like cysteine rich domain, a transmembrane domain and a tyrosine kinase domain. In addition, *unplugged* contains a kringle domain.
MuSK mutants in mice exhibit a similar phenotype to zebrafish *unplugged* mutants in that they have a complete loss of prepatterned and synaptic AChR clustering as well as excessive axonal growth (DeChiara et al., 1996). In mice, MuSK expression is restricted to skeletal muscle fibers, with low expression levels in proliferating myoblasts turning to strong expression in differentiated fibers (Valenzuela et al., 1995). Zebrafish *unplugged/MuSK* is localized to myoblasts of the slow-twitch lineage with expression first detectable at the bud stage (10 somites) and persisting until late somitogenesis (Zhang et al., 2004).

Unlike MuSK, which has two known splice variants (Hesser et al., 1999), the *unplugged* locus encodes three splice variants: full length (FL), splice-variant 1 (SV1), and splice-variant 2 (SV2) (Zhang et al., 2004). SV2 does not appear to be involved in prepattern formation or axon guidance. *Unplugged* FL, like MuSK, is required for formation and stabilization of mature synapses. Its role in synapse formation was inferred from a series of experiments in which FL morphant embryos were found to have normal AChR prepatternig but no mature synapses. It is possible to re-establish normal synapses when FL is expressed in an *unplugged* null background (Jing et al., 2010).

*Unplugged* SV1, which lacks the three extracellular Ig domains found in FL, is expressed at the 10 somite stage, a full ten hours before onset of FL expression. Unlike FL, SV1 is required for formation of the prepattern but not for the formation of mature synapses as determined by selective SV1 morpholino-mediated knockdown. SV1 is also required for proper axon guidance. Both the prepattern and axon guidance defects can be rescued by expression of SV1 in adaxial cells early in development (Jing et al., 2010). Furthermore, complete rescue only requires *unplugged/MuSK* to be supplied in a subset of adaxial cells dorsal to the muscle pioneers (Zhang and Granato, 2000).
In mutants where only SV1 is affected, growth cones stray away from their narrow path along the center of muscle fibers and make guidance errors at the choice point by either stalling or branching laterally. Only CaP and RoP show abnormal pathfinding while MiP, which innervates the dorsal myotome, appears unaffected (Zhang and Granato, 2000). The mechanism underlying MiP’s normal pathfinding in unplugged mutants is unknown. Live imaging during axonal outgrowth has revealed that growth cones become splayed and disoriented as soon as they enter the muscle territory, suggesting that unplugged/MuSK is required to direct axonal outgrowth from the very first moment that the growth cone enters the muscle territory (Jing et al., 2009).

In general, growth cones are guided by the presence of attractive positive cues and repulsive negative cues in their surrounding environment. Previous members of the laboratory showed that unplugged/MuSK affects the localization of two repulsive guidance cues, Tenascin (Schweitzer et al., 2005) and Chondroitin Sulfate Proteoglycan (Zhang et al., 2004), in the central muscle zone. Unplugged/MuSK’s ability to coordinate AChR clustering on the muscle membrane with axonal guidance may be explained by its ability to form a central muscle zone that restricts receptor clustering and secretion of guidance cues, yet how unplugged/MuSK initially forms and maintains this zone is unknown.

A large number of downstream effectors of unplugged/MuSK signaling have been identified including Rapsyn, a membrane-associated protein that colocalizes with AChRs at synaptic sites (Gautam et al., 1995), Dishevelled (Luo et al., 2002) and Dok-7 (Okada et al., 2006), but a unifying model for how unplugged/MuSK achieves centralized AChR clustering and axon guidance has not been developed. Overexpression studies in mice and rats suggest that unplugged/MuSK has the ability to stochastically autoactivate and induce AChR clustering (Hesser et al., 1999; Jones et al., 1999; Kim et al., 2008b;
Sander et al., 2001) but this does not preclude the possibility of activation by in vivo ligands. In this thesis, I explore the contribution of the ligands \textit{wnt11r} and \textit{wnt4a} to the activation of \textit{unplugged/MuSK}.

\textbf{Wnt and Planar Cell Polarity Signaling}

Wnts are known to bind the cysteine rich domain (CRD) of the transmembrane receptor Frizzled, as Frizzled receptors lacking the CRD are not able to bind Wnt (Bhanot et al., 1996). Additionally, Wnts are known to bind the CRDs of many proteins in addition to Frizzled, including the orphan receptor tyrosine kinase Ror2 (Billiard et al., 2005; Hikasa et al., 2002; Yoda et al., 2003) and frizzled-related protein (FRP) (Leyns et al., 1997; Lin et al., 1997; Rattner et al., 1997).

The lab has shown that Unplugged/MuSK, like other CRD containing proteins, can bind Wnt and that this binding requires the CRD (Jing et al., 2009). Wnts are natural regulators of synapse formation, as evidenced by the large numbers of papers implicating Wnts in synapse formation in \textit{Drosophila}, \textit{C. elegans} and the mammalian central nervous system (reviewed in Davis and Ghosh, 2007; Speese and Budnik, 2007), as well as in neuromuscular junction development (Ataman et al., 2008; Li et al., 2008; Packard et al., 2002; Wang et al., 2003; Zhang et al., 2007a). Additionally, MuSK has been shown to directly interact with Lrp4, a protein related to the known Wnt binding Frizzled co-receptor Lrp5/6 (Kim et al., 2008b; Zhang et al., 2008). Lrp4 mutants in mice are unable to cluster AChRs, just like \textit{unplugged/MuSK} mutants (Weatherbee et al., 2006). Finally, a previous graduate student and I demonstrated that, in vivo, \textit{unplugged/MuSK} signaling requires the Wnt pathway component \textit{dishevelled} (Jing et al., 2009). These observations, combined with evidence presented in this thesis, strongly argue for a role for Wnt signaling in early neuromuscular junction development.
Binding of Wnt to Frizzled can trigger one of three downstream signaling pathways; the canonical pathway, the noncanonical calcium pathway, and the noncanonical planar cell polarity (PCP) pathway. In canonical signaling, binding of Wnt to Frizzled activates Dishevelled, which ultimately allows for translocation of β-Catenin into the nucleus where it interacts with TCF. Binding of β-Catenin to TCF allows for expression of a number of genes involved in cell fate and axis duplication (Montcouquiol et al., 2006). The two noncanonical pathways begin with binding of Wnt to Frizzled and activation of Dishevelled, but at this point they diverge and are no longer dependent on β-Catenin and TCF. The calcium pathway results in an increase in intracellular calcium, as well as activation of PKC and CamKII.

The noncanonical PCP pathway triggers the activation of the small GTPase Rho, which acts through the Rho-associated kinase (ROCK) to elicit changes in the actin cytoskeleton independently of transcription (Kim and Han, 2005; Marlow et al., 2002; Winter et al., 2001). The PCP pathway is credited with coordinating the structural orientation of a group of cells and convergent extension movements. For example, PCP directionally orients *Drosophila* wing hairs and symmetrically arranges ommatidia in the *Drosophila* eye (reviewed in Jenny and Mlodzik, 2006). Which Wnt signaling pathway is activated depends, in part, on which of the 19 vertebrate or 7 *Drosophila* Wnts binds to Frizzled (reviewed in Montcouquiol et al., 2006). For instance, in vertebrates, *wnt11r* and *wnt4a* are considered to be noncanonical pathway activators (Matsui et al., 2005), while *wnt1* and *wnt3* are usually canonical (Shimizu et al., 1997). It is important to note that in *Drosophila*, there is no compelling evidence for a direct interaction between Wnt and Frizzled that initiates PCP signaling, though Wnts remain attractive candidate ligands.
Together, these noncanonical signaling components have the ability to modify the actin cytoskeleton, which in turn is responsible for establishing the polarized morphology of a wide variety of cell types (reviewed in Strutt and Strutt, 2009). It is also known that clustering of AChRs at the neuromuscular junction is dependent on the integrity of the actin cytoskeleton (Lee et al., 2009; Pato et al., 2008). Given these two observations, I propose a model in which the centrally-localized complex of noncanonical Wnt signaling components promotes AChR clustering via modifications to the actin cytoskeleton.

**Wnts as regulators of synapse formation**

Although Wnts are best known for their involvement in embryonic patterning and cell fate decisions, their contributions to axon guidance and synapse formation have recently become appreciated. Wnt signaling, which can elicit profound changes in cytoskeletal organization, is a natural regulator of axonal arborization and morphology. For example, in one of the first studies linking Wnt signaling to axonal morphology, \( wnt7a \) – which is expressed in cerebellar granule cell neurons – influences axon spreading and branching of these neurons in culture (Lucas and Salinas, 1997).

Although there are many similar reports of Wnts affecting neuronal morphology in an autocrine manner, Wnt signaling can also be anterograde or retograde (reviewed in Speese and Budnik, 2007). In invertebrate synapse formation, Wnt signals are provided in an anterograde or autocrine manner only. For example, *Drosophila wnt5* is required for proper pre- and postsynaptic development, but expression of \( wnt5 \) only in presynaptic boutons and not in the postsynaptic muscle is capable of restoring \( wnt5 \) mutant synaptic defects (Liebl et al., 2008). However, in vertebrates, several examples of retrograde Wnt signaling have been reported. In one example, expression of \( wnt3 \) in
murine motor neurons affects arborization and connectivity of presynaptic sensory axons (Krylova et al., 2002).

At the neuromuscular synapse, Wnts have been reported to directly act on the postsynaptic muscle fiber. In mice, Wnt3a inhibits AChR clustering on the postsynaptic muscle fiber by downregulating the expression of the AChR-anchoring protein Rapsyn (Wang and Luo, 2008). In chicks, Wnt3 has the opposite effect in that it increases Agrin-induced AChR microclusters with the assistance of the noncanonical signaling component Rac1 (Henriquez et al., 2008). In a similar positive regulatory event, application of Wnt1 triggers an increase in MuSK promoter-reporter activity in cultured myotubes (Kim et al., 2003).

This thesis provides an additional example of Wnts directly acting on the postsynaptic muscle fiber, but unlike previous reports, I also identify a unique role for Wnts as indirect, retrograde regulators of motor axon growth through reorganization of the muscle fiber. Furthermore, we observed that transplanting a wnt11r mutant axon to a wild-type background eliminated axon guidance errors, which suggests that wnt11r is not required in the motor axon itself for the observed guidance behavior, though I am unable to eliminate the possibility of some wnt11r bypassing the muscle fiber and acting directly on the axon.

Wnt11r is expressed in the spinal cord and in the somitic tissue immediately dorsal to the spinal cord, which means it must diffuse a short distance in order to interact with adaxial muscle cells. Wnt4a, however, is expressed at low levels throughout the somitic tissue, including the area occupied by adaxial cells. This leaves open the possibility that wnt4a acts as an autocrine cue in that it is both secreted by and signaling to the same muscle fibers. However, additional experiments must be performed to clearly identify the source of the relevant Wnt signals.
I propose that binding of noncanonical Wnts to Unplugged/MuSK initiates a downstream signaling cascade that is reminiscent of PCP signaling. This PCP signaling could dictate polarity along the anterior-posterior axis of the muscle fiber through directed secretion of extracellular matrix proteins, thus defining a restricted band on the muscle fiber along which motor axons will extend. In the absence of PCP signaling, this specialized domain fails to form, and growth cones, which are no longer limited to a defined zone, explore a broader area and make incorrect guidance decisions. This specialized zone may also coordinate AChR prepattern formation in the center of the muscle fiber. It is possible that the other Wnt pathways may also play a role in axon guidance and AChR clustering, but activation of PCP signaling provides the strongest model and has yielded the most compelling data.

**Endocytosis as a means for enhancing receptor signaling**

Once thought to be a means for removing key signaling components from the membrane and targeting them for degradation, the endocytic pathway is now becoming appreciated for its ability to activate and/or stabilize these same components with the effect of increasing rather than quenching signaling (Gagliardi et al., 2008). A number of well-known signaling pathways have been shown to rely on endocytosis for receptor signaling. For instance, one group reported that a subset of epidermal growth factor receptors (EGFRs) rely on clathrin-mediated endocytosis to achieve its full repertoire of signaling activity (Vieira et al., 1996). Similarly, transforming growth factor β (TGFβ) receptor is endocytosed and then segregated into two groups, one of which is targeted for receptor degradation while the other activates downstream signaling targets (Di Guglielmo et al., 2003). Also, inhibition of Dynamin-mediated endocytosis has been shown to block Notch signaling by reducing uptake and activation of the Notch
extracellular domain (Parks et al., 2000). It was further proposed that acidification of endosomal compartments via the vacuolar proton pump V-ATPase is critical for proper Notch signaling and endosomal trafficking (Yan et al., 2009).

In a scenario similar to the endocytosis of unplugged/MuSK, it was shown that application of Wnt induces the internalization and activation of the Frizzled receptor (Chen et al., 2003). GFP-tagged Frizzled localizes to asymmetrically-tracking vesicles that express the early/recycling endosome markers Rab4/5, and these particles are no longer visible in the absence of dishevelled or prickle, two core Wnt signaling components (Shimada et al., 2006). In muscle fibers, unplugged/MuSK behaves like frizzled in its role as the primary Wnt receptor, and Unplugged/MuSK-positive vesicles are also abolished in a dishevelled knockdown scenario. Together, these results suggest that there is something special about the endosomal compartment, whether it is the acidic conditions or simply its ability to bring key signaling proteins in proximity to one another, that enhances receptor signaling.

**Endocytosis and unplugged/MuSK signaling**

Despite growing evidence for the involvement of Wnt signaling in synapse formation, how Wnt mechanistically organizes pre- and postsynaptic development is still unknown. Here, I report that Wnt11r/4a trigger the internalization of Unplugged/MuSK into endosomes located in the center of the muscle fiber, and that this internalization is critical for coordinating centrally-restricted AChR clustering and motor axon guidance. Although the finding that a receptor tyrosine kinase localizes to and signals from endosomes is somewhat surprising, it is consistent with previous findings that MuSK undergoes rapid endocytosis following treatment with Agrin, a ligand important for AChR clustering during later synaptic development (Zhu et al., 2008).
I propose a model in which Wnt4a/11 binding to Unplugged/MuSK – coupled with some baseline level of ligand-independent autophosphorylation – triggers endocytosis of the receptor into recycling endosomes. These Unplugged/MuSK containing Rab11-positive endosomes are enriched and actively maintained in the center of the muscle fiber. I propose that the C-terminus of Unplugged/MuSK, which likely protrudes into the cytoplasm, binds the Dishevelled DEP domain, an interaction that has been previously characterized in vitro (Luo et al., 2002). Dishevelled has also been found to associate with vesicles in other systems. For example, it was reported that localization of Dishevelled to endosomes positively correlates with Wnt signaling in Xenopus (Capelluto et al., 2002). Additionally, blocking endocytosis triggers rapid degradation of Dishevelled, which suggests that endocytosis is important for protein stability (Bryja et al., 2007). It should be noted that there are reports of the inverse movement of Dishevelled from vesicles to the membrane in the presence of the Wnt receptor Frizzled (Axelrod et al., 1998; Rothbacher et al., 2000), so the behavior of Dishevelled may vary depending on the system and complement of binding partners.

In addition to binding Unplugged/MuSK, the DEP domain of Dishevelled is also capable of binding the planar cell polarity pathway components Diversin/Ankrd6 (Moeller et al., 2006) as well as Daam1 (Habas et al., 2001), and I suggest that this interaction may be responsible for the observed centralized, punctate localization of the two proteins. Just like Dishevelled, Diversin/Ankrd6 and Daam1 have been reported to localize to puncta in other systems. For instance, Diversin/Ankrd6-RFP localizes to cortical puncta in response to Wnt signaling in the Xenopus ectoderm (Itoh et al., 2009). Daam1-GFP localizes to endocytic vesicles along with Dishevelled in cultured cells, and eventually localizes to and alters the cytoskeleton as part of a later signaling event (Kida et al., 2007). This last observation highlights the importance of the endosomal
localization of Daam1 in bringing it into proximity of other signaling proteins, as well as promoting functional cytoskeletal interactions.

Daam1 is thought to indirectly influence the cytoskeleton through binding to RhoA (Habas et al., 2001), a small GTPase known for its ability to modify the actin cytoskeleton through further interactions with the Rho-associated kinase (ROCK) and LIM kinase (Maekawa et al., 1999; Matsui et al., 1996). I report that knocking down RhoA function in muscle fibers causes a reduction in central AChR clustering, arguing for the importance of RhoA as an adaptor. Altogether, this extensive network of protein interactions may form the basis for a bridge between unplugged/MuSK and the actin cytoskeleton.

How modifications to the actin cytoskeleton might affect AChR clustering, the functional readout of unplugged/MuSK signaling in muscle fibers, remains unanswered. AChRs have been shown to associate with the actin cytoskeleton at the neuromuscular junction, and furthermore, AChR clustering is dependent on the integrity of the actin cytoskeleton (Lee et al., 2009; Pato et al., 2008). Additionally, it was reported that tyrosine phosphorylation facilitates the interaction of AChRs with actin (Dai et al., 2000), which is consistent with the previous finding that unplugged mutant muscle fibers show decreased phosphotyrosine staining (Zhang et al., 2004). Taken together, these observations demonstrate how unplugged/MuSK-mediated modifications to the actin cytoskeleton could easily translate into functional changes in the stability of AChR clusters.

Another outstanding question is why Unplugged/MuSK-positive endosomes are initially enriched in center of the fiber. To date, the question of how Wnt signaling establishes polarized tissues in other systems such as the Drosophila wing disk is similarly unresolved. I suspect that the specialization of the muscle’s central zone is not
the responsibility of a single protein, but that a small initial bias translates into a selfsustaining network of protein-protein interactions between Unplugged/MuSK, Dishevelled and other PCP signaling components.
CHAPTER 2

Wnt signals organize synaptic prepattern and axon guidance through the zebrafish *unplugged* /MuSK receptor

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Abstract

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 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 non-canonical dishevelled signaling in muscle fibers disrupts AChR prepatternning 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 and Burden, 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 prepatterning 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) and (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 \textit{wnt11r} is required to confine navigating growth cones to the center of muscle fibers, and to initiate AChR prepattern. We show that \textit{wnt11r} and \textit{unplugged} interact genetically, that Wnt11r binds Unplugged/MuSK through its \textit{frizzled}-like cysteine rich domain (CRD) \textit{in vitro}, that \textit{wnt11r} binding in the embryos depends on the presence of \textit{unplugged}/MuSK, and that non-canonical \textit{dishevelled} signaling in muscle fibers is required for \textit{unplugged}/MuSK function. Together, the data provide strong evidence that Wnt ligands activate \textit{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 somites (Flanagan-Stee 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-twitch muscle fibers invade the space on the medial somite surface (Cortes et al., 2003). Motor growth cones then contact medial fast-twitch muscle fibers, and form neural en passant synapses at sites previously marked by prepatterned AChRs (Flanagan-Stee et al., 2005; Panzer et al., 2005). A group of 2-5 non-migratory adaxial cells, termed muscle pioneers, remain on the medial myotome surface, and upon contact with motor growth cones, form the first neuromuscular junctions (NMJs) (Supplemental Fig. 1B Flanagan-Stee 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 (Fig. 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 (Fig.1 and Zhang et al., 2004). To determine which of the two unplugged/MuSK isoforms is critical to initiate adaxial AChR prepatternning 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 prepatternning, consistent with their differential expression patterns (Figure 1B-D). To confirm that the unplugged SV1 receptor is indeed responsible for AChR prepatternning, we generated transgenic lines in which myc-tagged unplugged SV1 is expressed under the control of a slow myosin heavy chain (smyhc1) promotor specific for adaxial cells (Fig. 1F-I 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.

unplugged/MuSK restricts growth cone migration to a central muscle zone

We then asked if unplugged/MuSK co-ordinates axonal pathfinding and AChR prepatternning. While we had previously shown that in the zebrafish embryo unplugged/MuSK is critical for axonal guidance after the period of AChR prepatternning (Zhang and Granato, 2000), we decided to examine axonal pathfinding at an earlier stage
during the period of AChR prepattern. For this, we used the Hb9 transgenic line, in which motoneurons express GFP (Flanagan-Steet et al., 2005). We imaged pioneering wildtype and *unplugged* mutant growth cones, as they exited from the spinal cord and entered the muscle territory, traversing the central muscle zone. In wildtype 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 fasiculates with CaP (Eisen et al., 1990). Confocal time lapse imaging confirmed that once they exited from the spinal cord, wildtype CaP and CaP/VaP growth cones pioneered a tight and narrow path (n=5 growth cones from five embryos, Fig. 2A, C and Suppl. Movie 1Eisen 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 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 non-canonical wnt11r ligand plays a role in AChR prepatternning 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 non-canonical Wnt family members known to induce such cellular polarity would be excellent candidates. Expression pattern analysis of published non-canonical 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 AChR patterning defects (Figures S2A-S2C).

Analysis of wnt11r mRNA expression in 20 somite stage embryos revealed strong signals in the spinal cord and in the dorso-lateral somites, just adjacent to unplugged/MuSK expressing dorsal adaxial cells, consistent with previously published data (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., 2009), was completely unaffected (Figures S2J and S2K). Instead, we observed two prominent phenotypes: axonal stalling and branching at 27hpf (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). Thus, morpholino knockdown of wnt11r causes unplugged/MuSK like 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 wildtype (Figures 3M-3Q; wnt11r MO: 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 (Figures 3M-3Q). 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 wildtype embryos, which induced unplugged like axonal defects in 13.3% hemisegments (n=977; Figure S3A). Injection of unplugged heterozygous embryos, which do not display any phenotypes, with such suboptimal wnt11r MO dose, significantly increased the number of axonal phenotype to 23.3% (n=1179; Supplemental Figure 3A). Moreover, using the same approach, we also observed an increase in AChR prepatterning defects, demonstrating that wnt11r and unplugged/MuSK interact genetically (Figure 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 might 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. Unplugged-GST fusion proteins, consisting of the unplugged SV1 extracellular domain tagged with GST (GST-SV1-ECD) were coupled to Glutathione-Sepharose, and then mixed with conditioned medium containing secreted Wnt11r-FLAG. Wnt11r proteins bound to Unplugged-GST 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 DCRD-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 SV1DCRD, 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), from where the injected protein is transported in the extracellular spaces throughout the entire embryos, and is exposed to the surface of all cells. In 20 somite stage wildtype embryos, we detected Wnt11r-FLAG binding on adaxial cells (Figure 4C). In unplugged mutant embryos binding of Wnt11r-FLAG was completely abolished (Figure 4D). Thus, 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 prepatter. Injection of mRNA encoding for wnt11r or unpluggedSV1 into wildtype
embryos revealed no difference in AChR prepatter (Figures 4E, 4F, and 4I). In contrast, co-injection of both wnt11r and unpluggedSV1 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 co-injected wnt11r and unplugged SV1DCRD (lacking the Wnt-binding domain), which did not increase AChR prepatter (Figures 4H and 4I). Thus, our results show that unplugged/MuSK and wnt11r are both are mutually required for induction of the AChR prepatter, 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 non-canonical 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 non-canonical 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 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 present (Figures 5C and 5D). Analysis of the AChR prepatter 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 results of blocking non-canonical 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, non-canonical Wnt signaling in adaxial cells is critical for axonal guidance and AChR prepatterning.

**Synapses form in the absence of AChR prepatter**

Our results show that *unplugged*/*MuSK* and *wnt11r* play critical roles in initiating the AChRs prepatter. These prepatterned AChR clusters can be incorporated into neuromuscular junctions, but is AChR prepatter essential for synapse formation? To answer this question, we generated multiple inducible *unplugged*/*MuSK* transgenic lines, in which the heat shock protein 70 (*hsp70l*) promotor (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 prepatter 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. 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 wildtype 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 H). 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 (Figure S6H).
Finally, expression of *unplugged*FL under the control of the adaxial specific promotor [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 prepatternning, when motor axons have extended well into the periphery (26 -somite stage, ~22hpf). Analysis of these embryos at 27hpf revealed the characteristic *unplugged* axonal defects, and the presence of neural AChR clusters (Figures 6I and 6J). Although these ‘rescued’ AChR clusters were abundant, there were smaller in size and less precisely aligned with the axons, when compared to wildtype (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 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., 2007a).

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., 2008b; 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 prepatternning defects, as well as unplugged like axonal defects already as growth cones navigate towards 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 to
adaxial muscle depends on unplugged function, suggesting that Unplugged/MuSK has
the properties of a wnt11r receptor. Fourth, blocking the dishevelled dependent non-
canonical 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 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 pre-
migratory dorsal adaxial cells in which unplugged function is required (Zhang and
Granato, 2000). Interestingly, Wnt11r secreted from these dorso-lateral somitic cells affect
AChR prepatterning of 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 50μm in 30 minutes 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 prepatterning is dispensable for NMJ formation**

In zebrafish, prepatterned AChR clusters are 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 is 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 strongly suggests that AChR prepatterning is dispensable for subsequent synapse formation. However, we can not 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 \textit{unplugged}/MuSK protein from the \textit{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’ \textit{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 site of synaptogenesis, while 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 \textit{unplugged}/MuSK, the organization of a central muscle zone is essential to restrict growth cones, as “late” \textit{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.

\textbf{The role of \textit{unplugged}/MuSK in synapse formation}

What is the role of \textit{unplugged}/MuSK in presynaptic development? In MuSK\textsuperscript{--/}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). 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 unplugged mutants, 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, only overexpression of Wnt11r and Unplugged induces ectopic AChRs, suggesting that wnt11r by itself is not sufficient to induce AChR prepatterning (Fig. 4), but that additional, wnt11r independent mechanisms, e.g. the mechanism to localize the unplugged/MuSK receptor, are also critical. Based on our data, we propose a model by which Wnt through unplugged/MuSK in muscle during the early phase of synaptogenesis activates a dishevelled signaling pathway, similar to the planar cell polarity pathway to define the positions 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 cones 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, Tenascin and Chondroitin sulfate proteoglycans (CSPG), 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., 2004; 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 becomes ligand independent due to a positive feedback loop (Jones et al., 1999; Moore et al., 2001). Elevating MuSK expression in 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 the first 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.
Materials and Methods

**Whole-mount immunocytochemistry**

Embryos were fixed and stained as described in (Zeller et al., 2002). For labeling of AChRs, embryos were permeabilized in 1mg/ml collegenase (Sigma) in phosphate buffer for 6-8 minutes, rinsed in 1xPBS and incubated with AlexaFluor conjugated α-bungarotoxin (Molecular Probes, Eugene, OR) as described by (Lefebvre et al., 2004). Antibodies and dilutions were used as follows: znpt1 (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.

Quantification of AChR clusters: Confocal images were projected into a single plane using the maximum projection and converted to a 16-bit image using Metamorph software. 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**

3-4 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. 6-7 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’-TCCTCACATTCCCTGCTCTGTGTC-3’ (forward) and 5’-
TCTTCATCTTCATTTGGGGCATC-3′ (reverse). mRNA was in vitro transcribed from linearized constructs using SP6 mMessage mMachine Kit (Ambion). mRNA was diluted in DEPC-H₂O and 0.1M KCl plus 0.5% phenol red and injected into embryos at the 1- to 2-cell stage.

**In vitro GST pull-down assay**

293T cells were grown in DMEM supplemented with 10% FBS, 100ug/ml of penicillin and streptomycin, and 2mM glutamine in a 37°C incubator with 5% CO₂. Cells were seeded in 10cm dishes from 1:10 split twenty-four hours before transfection. Cells were transfected with 8 µg of pCDNA-wnt11r-FLAG plasmid or pCDNA plasmid using Effectene Transfection Reagent (Qiagen) and grown for four days. The medium was then collected and concentrated using an Amicon Ultra-15 10K centrifugal filter device (Millipore). GST proteins and GST-UnpSV1ECD fusion proteins were expressed in *E.coli* and absorbed to Glutathione Sepharose 4B using the Bulk and RediPack GST purification Modules according to manufacture’s instructions (GE healthcare). Sepharose coupled with GST proteins were then incubated with medium containing wnr11rFLAG or control medium (pCDNA) for 2 hours at 4°C. Sepharose beads were washed four times with 1xPBS and bound proteins were eluted with 2x sample buffer. Eluted proteins were resolved on SDS-PAGE (10%) and blotted with anti-FLAG antibody (1:1000, Sigma) and anti-GST antibody (1:5000, Sigma). The secondary antibodies were HRP-conjugated goat anti-mouse and goat anti-rabbit (1:5000, GE healthcare). The blots were detected by ECL Plus chemiluminescent detection system (GE healthcare).

**Transient transfection, co-immunoprecipitation, and western blotting**
Transient transfection and immunoprecipitation were carried out as previously described (Lu et al., 2004) with some modifications. About 4x10^6 293T cells were seeded in 10 cm plate and grown for 24 hours at 37°C. 8µg DNA were transfected using Effectene Transfection Reagent, including 4 µg of pCDNA-wnt11r-FLAG and 4 µg of pcS2-SV1-myc or pcS2-SV1ΔCRD-myc. 48 hours post transfection, cells were lysed in 1 ml ice-cold lysis buffer (25mM Tris pH7.5, 150mM NaCl, 5mM EDTA, 1% Triton-X 100, 10% glycerol, 0.1% SDS, 1mM sodium orthovanadate, protease inhibitors (Sigma) and phosphatase inhibitors II (Sigma). Cell lysates were centrifuged at 14000rpm to remove insoluble materials and incubated with 10µl anti-myc agarose (Covance) for 2-3 hours at 4 °C. The immunoprecipitates were washed extensively four to five times using lysis buffer and eluted with 2x sample buffer and separated by SDS-PAGE (10%). Primary antibodies used were anti-FLAG (1:1000, Sigma), anti-myc (1:1000, Covance). Primer pairs 5’AAAAGAATTCCCATGATCAGGCCTGCAGACTCTC3’ (forward) and 5’AAAAGAATTCATTGAGTAGGCCGTAGACACAG3’ were used to clone the extracellular domain of SV1 (residues 1-299) to the EcoRI site of pGEX-2TK (GE healthcare) to make pGEX-UnpSV1ECD.

Transgenes

Myc-tagged SV1 or FL was cloned under the control of the zebrafish hsp70l promoter. To generate Tg (hsp70l:SV1-myc or hsp70l:FL-myc), 30 pg of supercoiled DNA was injected into unp^br307/+ embryos at the one cell stage. Their progeny were screened with anti-myc immunostaining for the expression of transgene following heat induction. The founder fish were then crossed to unp^br307br307 to create unp^br307br307; hsp-SV1(or FL)-myc/+ transgenic lines. Three independent lines were established for each transgene and were used to verify the results. The expression of transgene was detected within 15
minutes after heat induction for 35 minutes at 38°C. To generate transgene Tg(smyhc1:SV1-myc), myc-tagged SV1 was cloned under the control of the smyhc1 promoter (Elworthy et al., 2008). The transgene was flanked by an I-SceI site on both sides (opposite direction) and the injection was carried out as originally described in (Thermes et al., 2002). 30ng of DNA and I-SceI meganulcease were injected into one-cell-stage unp<sup>tbr307</sup>/+ embryos. The same procedure as above was used to make the Tg(unp<sup>tbr307</sup>tbr307; smyhc1:SV1-myc). The lines used in this studies are: Tg(hsp<sup>70</sup>l:unpSV1-myc)p1, Tg(hsp<sup>70</sup>l: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<sup>tbr307</sup>tbr307; hsp<sup>70</sup>l:SV1(FL)-myc/+ to unplugged<sup>tbr307</sup>tbr307 were kept at 28°C to the desired stage before the heatshock. Each pair of embryos was then placed in 100 µl E3 medium in a single well of 96-well PCR plate. Embryos were heat-shocked at 38°C for 35 minutes at 2.5 hours 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**

16- to 20-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 minutes using a Perkin Elmar UltraView spinning disk confocal equipped with a 63x lens. Growth cones were analyzed based on their morphologies during pathfinding.
**In vivo Wnt staining**

15-somite stage wild type and *unplugged* mutant embryos were injected in the yolk sac with 7nl of purified Flag-tagged Wnt11r protein, as previously reported for aBTX (Lefebvre et al., 2004). At the 20 somite stage were fixed in 4% PFA overnight, washed 2 x 10 minutes in PBST, briefly rinsed in water and then transferred into MeOH overnight at -20°C. Embryos were transferred into PBST for 5 minutes, fixed for 20 minutes at room temperature with 4% PFA, rinsed with PBST and blocked for 4 hours at room temperature in 2% Blocking Reagent (Roche) in MABT. After overnight incubation with anti-FLAG M2 alkaline phosphatase conjugate antibody (1:1000, Sigma-Aldrich), embryos were washed in MABT, incubated with staining buffer containing BCIP and NBT.

**Plasmid construction**

SV1 and FL constructs: *unpSV1* or *unpFL* full-length cDNA was cloned into expression vector pCS2+. To make C-terminally tagged *unplugged* constructs, a 5X myc tag was cloned into the NheI site of pcS2-SV1 or pcS2-FL to make pcS2-SV1-myc or pcS2-FL-myc. The endogenous signal peptide for each gene was retained. Two BsrGI sites were used to delete residues 32-173 (corresponding to the CRD) to make pcS2-SV1ΔCRD-myc. A ClaI/Apal digestion was used to move SV1-myc from pcS2-SV1-myc to the downstream of the *hsp70l* promoter (Halloran et al., 2000) to generate pBS-*hsp70l*:SV1-myc. A BstBI/SphI digestion of pCS2-FL-myc was used to replace the fragment digested by BstBI/SphI in pBS-*hsp70l*:SV1-myc to generate pSK-*hsp70l*:FL-myc.

Primer pairs 5’AAAAGAATTCCCATGATCAGGCCTGCAGACTCTC3’ (forward) and
5’AAAAGAATTCATTGAGTAGGCCGTAGACACAG3’ were used to clone the extracellular domain of SV1 (residues 1-299) to the EcoRI site of pGEX-2TK (GE healthcare) to make pGEX-\textit{UnpSV1ECD}. Primer pairs
5’AAGGTCTAGACCTCTCAGAACCATGATCGGCTGC3’ (forward) and 5’CTCACTATAATTTCTAGAGATCAGACGCCTGC3’ (reverse) were used to move SV1-myc into the XbaI site of the ISce1-smyhc1 vector to generate \textit{smyhc1:SV1-myc}. Primer pairs 5’AAAATCTAGAGTTCTGATACGAGGCTGACC3’ (forward) and 5’CTCACTATAATTTCTAGAGATCAGACGCCTGC3’ (reverse) were used to move FL-myc into the XbaI site of the ISce1-smyhc1 vector to generate \textit{smyhc1:FL-myc}.

\textbf{Wnt11r constructs:} Full-length \textit{wnt11r} cDNA was cloned into the expression vector pSK+ (a gift from Dr. Area Leham?). The PCR primers
5’TGTGGAGAAATACGTCTGCAAAGGATCCTGAGCTACTGGAC3’ (forward) and 5’GTCCAGTAGCTCAGGATCCTTTGCAGACGTATTTCTCCACA3’ (reverse) were used to remove the \textit{wnt11r} stop codon and 3’UTR in PBS-\textit{wnt11r} to generate PBS-\textit{wnt11rNS} (NS: non stop). 5’TTTTTGAGGATCCCACGTGACGTATTTTCTCC

ABC3’ (forward) and 5’TGGAGAAATACGTCTGCAAAGGATCCTGAGCTACTGGAC3’ (reverse) were used to remove the \textit{wnt11r} stop codon and 3’UTR in PBS-\textit{wnt11rNS} to form PBS-\textit{wnt11r-FLAG}. A BstXI/Apal fragment was then cloned into pCDNA3 to give rise to C-terminally tagged \textit{wnt11r} construct: pcDNA-\textit{wnt11r-FLAG}.

\textbf{Dsh constructs:} Primer pair: 5’AAGGTCTAGAGGATCCCATCGATTAAACCATG3’ (forward) and 5’ACGACTCTAGACTTTCTAGAGGCTCGAGG3’ (reverse) were used to add XbaI site to myc-Xdsh+\textit{DEP} from pCS2-myc-Xdsh-\textit{DEP+} (a gift from Dr. Peter Klein) and this PCR product was cloned into pCR-BluntII-TOPO (invitrogen). \textit{Myc-Xdsh-DEP+} was then cloned into the XbaI site of ISce1-smyhc1 to generate \textit{smyhc:myc-Xdsh-DEP+}.
Unplugged SV1 specific probe: 5’TGTATTATTGTTGTATCTGA
ACTTTTG3’(forward) and 5’GTGCTGCAGTAGCCGGCAT3’(reverse) were used to RT-PCR SV1 fragment (nt 1-340) from cDNA template made from 20-somite embryos and subcloned into pCR-BluntII-TOPO vector to make TOPO-SV1US (US: unique sequence). Unplugged FL specific probe:
5’GCCCGTTACTATTGAAGTACAAG3’(forward) and 5’TGTGC TGCAGTAGCCGGCATGG3’(reverse) were used to move FL fragment (nt 664-1012) to pCR-BluntII-TOPO to make TOPO-FLUS.

Constructs for yeast two-hybrid assay:
5’AAAAAGGATCCGGGTGCTGGAGACACCTACATTGAC3’ (forward) and 5’AAAAGGATCCTCAGCTAGCAGAAAGACCAGATTGAGCATC3’ (reverse) to subclone SV1 intracellular domain (IC, a.a.336-676) into pGBK7 (Clonetech).
A EcoRI/KpnI digestion of pME18S-zDsh2 (RZPD) was used to move full length of zDsh2 to pBSSK+(Stratagene). A EcoRI/Xbal digestion of pMES18S-zDsh3(RZPD) was used to move full length zDsh3 to pBSSK+. Primer pair: 5’AAAAAGGATCC TCATGAGCAGTGAA3’ (forward) and 5’AAAACTCGAGCATGATGTCGACG AAG3’ (reverse) was used to move zDsh3 (residues:185-676, containing PDZ and DEP domain) to pGADT7 (Clonetech). 5’ AAAAGAATTCGTGATGAGCAGTGAA3’ (forward) and 5’AAAA ATCGATACATCACATCCACAAAAAAC3’(reverse) was used to move zDsh2 (residues: 184-747, cotaining PDZ and DEP domain) to pGADT7.

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.

Two-hybrid interaction
pGBK7-SV1IC and pGADT7-zDsh2/3 were cotransformed into yeast AH109 strain and plated on SD/-Ade/-His/-Leu/-Trp plates with the addition of 15mM 3-AT according to manufacture’s instructions (Clonetech). The plates were incubated at 30°C until colonies appeared.

Purification of Wnt11r-FLAG HEK 293T cells (100mm dish) were transfected with 8µg of pcDNA-wnt11r-FLAG using the Effectene Transfection Reagent (Qiagen), and grown according to the manufacturer’s instructions for 5 days. The supernatant was concentrated to 1ml using an Amicon ultra-centrifugal filter unit (Sigma-Aldrich), added to 20µl of anti-FLAG M2 agarose (Sigma-Aldrich) that had been pre-washed with TBS and glycine HCL according to the manufacturer’s instructions. After overnight incubation at 4°C, the agarose was washed with TBS and Wnt11r-FLAG protein was eluted using 100µl glycine HCL pH 3.5 plus 0.5% Sodium Deoxycholate. Beads were centrifuged and the supernatant was collected and combined with 5µl of 0.5M Tris-HCl pH 7.4, 1.5M NaCl and stored at -20°C.
Figures and Legends

Figure 2.1 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 wildtype 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-G) Lateral views and cross-sectional views (H, 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.
Figure 2.2 *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, B), or CaP/VaP pair axons (C, D) from the spinal cord into the myotome. Arrows point to the single wildtype CaP growth cone (A) and to the tightly fasciculated wildtype 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 wildtype. Asterisks indicate interneurons also labeled by the Tg(*Hb9*:GFP).
**Figure 2.3 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, twenty hemisegments were analyzed; n=hemisegments. Results are expressed as the mean of multiple injection experiments ±s.e.m., (*p<0.0001, t test). (C-L) Wildtype, unplugged and wnt11r MO injected embryos at 27hpf (C-H), and at the 20-somite stage (I-L), stained for motor axons (znp-1, green) and AChR clusters (α-BTX, red). (E, F) In contrast to wildtype, unplugged embryos display characteristic stalling (arrowhead) and branches (arrows) at the choice point, and lack all AChR clusters. (G, H) Injection of wnt11r MO causes unplugged like axonal stalling (arrowhead), branching (arrow), and a strong reduction of AChR prepatterning (K, 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 wildtype (M, O) and wnt11r morphant CaP and VaP axons (N, P, Q), as they exit from the spinal cord (M, N), and as they reach the somitic choice point (O-Q). Asterisks indicate the cell body of interneurons. (M, O) Wildtype 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, Q), compared to wildtype (M, O). Scale bars: 50 μm.
Figure 2.4 Wnt11r binds to UnpSV1 and overexpression of wnt11r and unpSV1 increases 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 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). The bottom shows the schematic diagrams of constructs used in the experiments. SS: signal sequence. (C and D) Cross sections of 20-somite embryos injected with purified Wnt11r-FLAG protein. (C) In wildtype embryos, Wnt11r binds to adaxial cells as highlighted by the brackets. Binding is abolished in unplugged mutants (asterisks in D mark non-specific staining). (E-H) Wildtype embryos were injected with mRNAs as indicated. The domain of AChR prepatterning (brackets) was expanded in embryos co-injected with wnt11r and unpSV1myc mRNAs, and was dependent on the CRD domain. (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). The size of AChR clusters was also analyzed, but no significant difference was
observed between different groups. Amounts of mRNA (ng/embryo): \textit{wnt11r-FLAG}, 0.3; \textit{SV1myc}, 0.5; \textit{SV1ΔCRDmyc}, 0.5.
Figure 2.5 Inhibition of the non-canonical 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 6-7 adaxial cells (in grey) used for scoring. (D) Analysis of axonal phenotypes. (n=hemisegments; blue, hemisegments with 2 adaxial cells expressing the transgene; yellow, hemisegments with 3 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 from 20-somite stage embryos. 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 prepattern between transgene expressing cells (F’, open arrowhead); note that this does not affect adjacent, non-transgenic cells which formed normal AChR clusters (F’, arrows). For each transgene, four embryos with GFP or Myc-Dsh-DEP+ positive adaxial were analyzed. Prepattered clusters were reduced in all Myc-Dsh-DEP+ expressing embryos. Scale bars: A, 50 μm; E, 10 μm.
Figure 2.6 Neuromuscular synapses form in the absence of AChR prepatter.

20-somite stage (A-B, G-H) or 27 hpf (C-F and I-J) embryos after heat shock treatment. (A, B) Tg(hsp70l:UnpSV1-myc; unplugged) embryos received heat shock from the 10- to 20-somite stage, which rescued AChR prepatter. (C, D) Similar heat shock treatment (10-somite to 27 hpf) also restored motor axon pathfinding, but not neuromuscular synapses. (E, F) The same heat shock treatment rescued motor axons and neuromuscular synapses in Tg(hsp70l:UnpFL-myc; unplugged) embryos. (G, H) In contrast, heat shock between the 10- and 20-somite stage failed to rescue AChR prepatter in Tg(hsp70l:UnpFL-myc; unplugged) embryos. (I, 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 μm.
Figure 2.7 unplugged/MuSK signaling during synapse formation.

Signaling during the early (A, B) and late (C, D) phases of neuromuscular synapse formation. (A, B) Early during synapse formation, Wnt signals act through unplugged/MuSK receptor to establish a central muscle zone, possibly through a Dsh-dependant, PCP like pathway. LRP4 is essential for MuSK localization, and Dok-7 for enhancing 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 mechanisms, 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, 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 form, but that local agrin secretion from the axon and late expression of unplugged/MuSK appears sufficient to induce neural AChRs and subsequently functional synapses.
EARLY

wnt signals (neural tube, lat. somite)
LRP4 (MuSK localization)
unplugged/MuSK
(Dok-7)
Dsh
central zone (via PCP-like pathway?)
Rapsyn
ECM modifications
(CSPGs, Tenascin)
ACHR prepatter
growth cone guidance

LATE

Agrin from axons
unplugged/MuSK
LRP4
Dsh?
Dok-7
Rapsyn
neural AChRs
synapse
Supplemental Figure 2.1 *Unplugged* full-length (FL) and Splice Variant 1 (SV1) are differentially expressed.

(A-B) Schematic representation of AChR clustering. (A) Prior to the arrival of growth cones, migratory adaxial cells (light green) flank non-migratory adaxial or muscle pioneer cells (dark green, MP) and form a monolayer along the medial somite. Prepatterned AChR clusters (red) accumulate on the medial surface of all adaxial cells. As the first growth cones (blue) approaches, migratory adaxial fibers (light green) initiate their radial migration to the lateral surface of the somite, while fast muscle fibers (peachy) invade the space on the medial somite surface. NC, notochord. (B) Growth cones contact fast fibers and form neural *en passant* synapses. At the horizontal midline, growth cones contact muscle pioneers and incorporates prepatterned clusters into NMJs. (C-F) Confocal images of cross-sections from 20-somite stage wildtype embryos double stained for *unplugged* mRNA and F59 antibody, specific for adaxial cells. Just before the first motor axon exit from the spinal cord, *unplugged* SV1 is highly expressed in all pre-migratory adaxial cells (C-D), while *unplugged* FL is expressed at a much lower level (E-F) compared to *unplugged* SV1. Scale bar: 50 μm.
**Supplemental Figure 2.2 wnt5/11 mutant analysis, wnt11r expression and stainings of wnt11r morphants.**

(A) In 27 hpf wildtype embryos, neural AChR clusters (red, α-BTX) are apposed to primary motor axons (green, znp-1). In *silberblick* (*slb*/wnt11, B) and *pipetail* (*ppt*/wnt5a, C) mutants, axon pathfinding and neuromuscular synapses are unaffected. (D-G) Cross-section images from 20-somite stage wildtype embryos double stained for *wnt11r* mRNA (green) and adaxial cells (red, F59). In caudal segments (D and E), *wnt11r* is expressed in the spinal cord and in the dorso-lateral somites, just adjacent to pre-migratory adaxial cells. In the rostral segments (F and G), after the onset of adaxial cell migration, *wnt11r* expression is increased in the spinal cord. Dashed lines indicate the spinal cord. (H and I) Confocal images of embryos at 26-somite stage stained for CSPs (red) and motor axons (green). CSPs accumulate around the choice point (brackets) in wildtype embryos (H), but are reduced in *wnt11r* morphants (I). (J-K) Lateral views of adaxial fibers in wildtype and *wnt11r* MO-injected embryos. Scale bars: 50 μm.
Supplemental Figure 2.3 Genetic interactions between *unplugged* and *wnt11r*.

(A) Quantification of motor axon defects at 27 hpf using sub-optimal *wnt11r* TL-MO dose (2 ng). *unplugged*/+ embryos are indistinguishable from wildtype embryos. The same amount of sub-optimal dose of *wnt11r* TL-MO injected into wildtype embryos resulted in 13% *unplugged*-like axonal defects, while injection into *unplugged*/+ embryos increased the phenotype to 23%. Twenty hemisegments were scored in each embryo; n=hemisegments. Results are compiled from multiple experiments as the average±S.E.M. (t-test, *p<0.01). (B-C) Analysis of prepatternning phenotype using optimal *wnt11r* TL-MO concentrations (3-4 ng) . (B1-B3) Each hemisegment of embryos was scored as normal prepatternning, mild reduction or strong reduction. (C) Quantification data show prepatternning phenotype was increased in *unplugged*/+ embryos. Results were obtained from three different experiments; n= hemisegments. 3 or 4 hemisegments were scored in each embryo.
Supplemental Figure 2.4 Coimmunoprecipitation of UnpFL with Wnt11r in 293T cells and analysis of the Tg(smyhc1:UnpFLmyc) embryos. (A) 293T cells were cotransfected with Wnt11r-FLAG and UnpSV1-myc or UnpFL-myc. Whole cell lysates were subjected to anti-FLAG immunoblotting (IB) to determine the expression of Wnt11r-FLAG (lower panel). The Wnt11r-FLAG binding was assessed by IB of the anti-myc immunoprecipitate (upper panel). The amount of UnpFL or UnpSV1 proteins were examined by anti-myc western blotting. Wnt11r-FLAG protein coimmunoprecipitated significantly better with UnpSV1 compared to UnpFL. (B) Cross-sectional view of a 17 hpf Tg (smyhc1:unpFLmyc) embryo stained with anti-myc. (C) Expression of UnpFL in adaxial cells failed to restore prepatterned AChRs (red) in unplugged embryos.
Supplemental Figure 2.5 Unplugged interacts with zebrafish Dishevelled.

(A) The intracellular domain of unplugged /MuSK (Unpic) was cloned into a bait plasmid pGBKT7. The PDZ and DEP domains of zebrafish dishevelled 2 and dishevelled 3 were cloned into a prey plasmid pGADT7. Two-hybrid interactions were performed using SD/-Ade/-His/-Leu/-Trp media to screen for reporter genes: ADE2 and HIS3 (+++: fast growth. ++: intermediate growth. -: no growth). (B, C) The morphology and differentiation of adaxial cells expressing Myc-Xdsh-DEP+ appears normal. 28 hpf embryo injected with symhc1:myc-Xdsh-DEP+ was stained with mAb anti-Myc (red) and F59 (green, adaxial cells).

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Supplemental Fig. 5 Jing et al
Supplemental Figure 2.6 Formation of neural synapses can occur independently of AChR prepatternning.

(A-D) Images of untreated Tg(hsp70l:UnpSV1-myc;unplugged) embryos (A, B), and Tg(hsp70l:UnpFL-myc; unplugged) embryos at the 20-somite stage (A, C) or at 27 hpf (B, D). AChR prepattern and neural synapses (red, α-BTX) are absent, and axons display unplugged-like phenotypes (green, zn-1). (E-G') High-magnification views of neural synapses in wildtype embryos (E) and 'rescued' transgenic embryos. Embryos heat-shock treated between the 26-somite stage and 27 hpf, displayed unplugged-like axons with neural AChRs apposed the length of the axon (G, G'). (H) Cell extracts from individual transgenic embryos, with and without heat shock treatment (38ºC for 35 min), were subjected to anti-myc immunoblotting. Scale bars: in C and D, 50 μm; in E, 20 μm.
CHAPTER 3

Initiation of synapse formation by Wnt-induced

Unplugged/MuSK endocytosis

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Running title: Wnt induced MuSK endocytosis initiates NMJs

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Abstract

The MuSK receptor initiates neuromuscular synapse formation by restricting presynaptic growth cones and postsynaptic acetylcholine receptors (AChRs) to the center of vertebrate muscle cells. Increasing evidence suggests a role for Wnts in this process, yet how muscle cells respond to Wnt signals is unclear. Here, we show that in-vivo, \textit{wnt11r/wnt4a} initiate MuSK translocation from muscle membranes to recycling endosomes, and that this transition is critical for AChR localization to future synaptic sites. Moreover, we demonstrate that components of the planar cell polarity pathway colocalize to recycling endosomes, and that this localization is MuSK dependent. Knockdown of several core components disrupts MuSK translocation to endosomes, AChR localization and axonal guidance. We propose that Wnt-induced trafficking of the MuSK receptor to endosomes initiates a signaling cascade to align pre- with postsynaptic elements. Collectively these finding suggest a general mechanism by which Wnt signals shape synaptic connectivity through localized receptor endocytosis.
Introduction

Synapse formation requires the precise alignment of pre- and postsynaptic elements. In the case of neuromuscular synapses, this process involves proper navigation of motor axons from the spinal cord to their muscle targets as well as the spatial alignment of these motor axons with acetylcholine receptors (AChRs) on the muscle surface (reviewed in Wu et al.). The process of neuromuscular synapse development can be subdivided into two functionally distinct phases, an early and a late phase (Flanagan-Steet et al., 2005; Panzer et al., 2006; Wu et al.; Zhang et al., 2004). The late phase is characterized by the transformation of motor growth cones into presynaptic nerve terminals which release signals, including Agrin, that are essential for postsynaptic stabilization and maturation (Campanelli et al., 1991; Godfrey, 1984; Ruegg et al., 1992; Wu et al.). In contrast, the early phase is independent of the nerve and nerve-derived factors (Lin et al., 2001; Yang et al., 2001; Yang et al., 2000). During the early phase, even before the arrival of motor axons, AChRs accumulate in the center of muscle cells, precisely where the first synapses will form in a process termed AChR prepatterning (Lin et al., 2001; Yang et al., 2001). Importantly, ‘blocking’ AChR prepatterning through temporal inactivation of MuSK does not eliminate the formation of functional synapses, although synapses form at ectopic locations, suggesting that events during the early phase of neuromuscular development determine where synapses will form (Jing et al., 2009).

A key player in determining where along the muscle cell synapses will form is the receptor tyrosine kinase muscle specific kinase, MuSK (DeChiara et al., 1996; Glass et al., 1996). In both mice and zebrafish, MuSK is expressed in early postsynaptic muscle cells, and in embryos lacking MuSK or its zebrafish ortholog unplugged, AChRs fail to cluster in the center of muscle cells, and motor axons invade lateral muscle
territory they usually avoid (DeChiara et al., 1996; Lin et al., 2001; Yang et al., 2001; Zhang et al., 2004). MuSK is sufficient to establish muscle prepatterning through ligand-independent autoactivation (Kim and Burden, 2008). At the same time, the secreted glycoprotein Wnt11r binds to the Unplugged/MuSK ectodomain, and functional knockdown of wnt11r leads to defects in AChR prepatterning and motor axon guidance (Jing et al., 2009). While these studies suggest that Wnt signals play a critical role in vertebrate neuromuscular development, the functional requirement for Wnt has not been confirmed using genetic mutants, and the mechanism by which Wnt signals initiate synapese formation has not been established.

Here, we characterize a null mutant for wnt11r and show that wnt11r and wnt4a are essential for triggering relocalization of the Unplugged/MuSK receptor from the cell membrane to recycling endosomes located in the center of early muscle cells, precisely where future synapses will form. We provide compelling evidence that Unplugged/MuSK localization to recycling endosomes activates a signaling cascade best known for its role in mediating planar cell polarity (PCP). Furthermore, inhibition of selective PCP components results in a reduction of AChR prepatterning and axon guidance errors. We propose a model in which rab11-mediated endocytosis positions a signaling complex consisting of the Unplugged/MuSK receptor and PCP components to the center of muscle cells to initiate synapse formation.
Results

\textit{wnt11r and wnt4a are required for synaptic development}

We previously reported that Wnt11r binds the Unplugged/MuSK receptor, and that morpholino-mediated knockdown of \textit{wnt11r} recapitulates the pre- and postsynaptic defects observed in \textit{unplugged} null mutants (Jing et al., 2009). These results suggest that Wnt11r is a ligand for Unplugged/MuSK, yet the incomplete phenotypic penetrance observed in \textit{wnt11r} morphants left open the possibility of a partial morpholino knockdown, maternal contribution and/or additional ligands. To distinguish between these possibilities, we characterized embryos carrying an early stop codon in \textit{wnt11r}, likely to represent a \textit{wnt11r} null allele (G94*) (Banerjee et al., submitted). We found that in 100\% of \textit{wnt11r} mutant somitic segments, the AChR prepattern was no longer restricted to the center of muscle cells as is the case in wild-type embryos (Figure 1A), but instead spread over a broader area (~61\%; Figure 1A and 1E and quantified in 1L) or was completely dispersed (~39\%; Figure 1F). Likewise, in many somitic segments (26\%), motor axons occupied a broader area, identical to the phenotype observed in \textit{unplugged} mutants (Figure 1B and 1D and 1G). Importantly, overall muscle morphology in \textit{wnt11r} null mutants was indistinguishable from wild-type siblings (Figure S1A and S1B). Moreover, embryos derived from \textit{wnt11r} mutant mothers displayed identical AChR and axonal phenotypes at similar frequencies (data not shown), suggesting that maternal \textit{wnt11r} contributions are dispensable for synapse initiation. Thus, \textit{wnt11r} null mutants recapitulate the pre- and postsynaptic defects observed in \textit{unplugged} null mutants (Figure 1C-1D), albeit with lower penetrance, suggesting the existence of additional \textit{unplugged}/MuSK ligands.

Given the great extent of redundancy among vertebrate Wnts, we hypothesized that other Wnts might be compensating in the absence of \textit{wnt11r}. Based on its mRNA
expression pattern in the vicinity of muscle cells that first form the AChR prepattern, we focused on \textit{wnt4a} (Figure S1E-S1F). Using a previously characterized translation-blocking morpholino (Matsui et al., 2005), we found that knockdown of \textit{wnt4a} by itself did not produce detectable perturbations of AChR prepatterning or axon guidance (Figure 1H-1I). In contrast, injection of the same dose of \textit{wnt4a} morpholino into \textit{wnt11r} mutants exacerbated the partial \textit{wnt11r} null phenotype, and in 100% of somitic segments, the AChR prepattern was either highly disorganized or else completely absent (Figure 1J). Concomitantly, in 70% of \textit{wnt11r}/\textit{wnt4a} deficient somitic segments, motor axons displayed \textit{unplugged}-like branching defects, again without affecting overall muscle cell morphology (Figure S1C-S1D). Thus, loss of \textit{wnt11r} and \textit{wnt4a} recapitulates the AChR prepattern and axonal defects observed in \textit{unplugged} null mutants (Zhang and Granato, 2000), consistent with the idea that, \textit{in vivo}, neuromuscular synapse formation is initiated by the \textit{unplugged}/MuSK receptor and multiple Wnt ligands.

\textbf{\textit{wnt11r} regulates the level of membrane-bound Unplugged/MuSK}

We next asked how \textit{wnt11r} and \textit{wnt4a} initiate synapse formation. We had previously shown that ubiquitous expression of Unplugged-Myc along the entire length of muscle cells did not alter the centrally localized AChR prepattern (Jing et al., 2010), suggesting that additional factors or processes might determine where along the muscle cell Unplugged/MuSK signals. In the early zebrafish embryo, Wnts recruit the Frizzled receptor and the obligate intracellular signaling component Dishevelled to the plasma membrane, thereby generating local cell contacts important for gastrulation movements (Witzel et al., 2006). To test if Wnts might alter the cellular distribution of Unplugged/MuSK, one-cell stage embryos were injected with mRNAs encoding GFP-tagged \textit{unplugged}/MuSK and the membrane marker mCherry-CAAX, in the absence or
presence of \textit{wnt11r} mRNA. Four and a half hours later at dome stage, injected embryos were analyzed for the cellular localization of Unplugged/MuSK-GFP protein (Figure 2). Importantly, these early embryos do not express \textit{wnt11r} endogenously, providing a “Wnt naïve” environment (Nojima et al., 2010).

In the absence of \textit{wnt11r} mRNA, Unplugged/MuSK-GFP is highly enriched at the cell membrane, as is expected for a receptor tyrosine kinase (Figure 2A-2A’’). In contrast, in the presence of \textit{wnt11r} mRNA, levels of Unplugged/MuSK-GFP at the cell membrane were dramatically reduced (Figure 2B-2B’’; quantified in E). Moreover, deletion of the cysteine rich domain (CRD), which is essential for Wnt11r binding to the Unplugged/MuSK-GFP ectodomain (Jing et al., 2010), did not affect insertion of Unplugged/MuSK-GFP into the membrane in the absence of \textit{wnt11r} (Figure 1C-1C’’), but reduced \textit{wnt11r} responsiveness (Figure 1D-1D’’; quantified in 1F). These data demonstrate that exposure to \textit{wnt11r} alters membrane localization of Unplugged/MuSK, which is consistent with the idea that \textit{wnt11r} either destabilizes Unplugged/MuSK, and/or causes translocation from the plasma membrane.

\textbf{Unplugged/MuSK-GFP localization to recycling endosomes ‘marks’ the site of future synapses}

To explore the relationship between Wnt exposure and Unplugged/MuSK localization in the relevant cellular context, we examined Unplugged/MuSK localization in early muscle cells, also called adaxial cells, which form the AChR prepattern. Unplugged/MuSK mRNA is detectable prior to and during the time period of AChR prepatterning in adaxial muscle cells, and becomes undetectable soon afterwards (Zhang et al., 2004). To express \textit{unplugged}/MuSK in adaxial muscle cells, we cloned 3.8kb of genomic DNA
directly upstream of the *unplugged/MuSK* translational start site (*3.8unp*) and used this to drive the expression of *GFP* or *unplugged/MuSK-GFP*.

Injection of a bona-fide adaxial muscle promoter (*smyhc1:GFP*) (Elworthy et al., 2008) or the *3.8unp* promoter driving *GFP* resulted in GFP expression throughout the cytoplasm of stochastically labeled adaxial muscle cells (Figure 3A, B). Stochastic labeling results in individual cells that retain and express the injected DNA construct, surrounded by cells which do not retain the DNA and hence do not express the construct (Downes et al., 2002). Injection of *3.8unp* driving *unplugged/MuSK-GFP* (*3.8unp:unplugged-GFP*) in *unplugged* null mutant embryos restored AChR prepatterning, demonstrating that this construct is functional (Figure S2B). Injection of *3.8unp:unplugged-GFP* into wild-type embryos resulted in stochastic GFP expression in adaxial muscle cells, but unlike GFP, Unplugged/MuSK-GFP localized to puncta in the center of adaxial muscle cells before and during the time period of AChR prepatterning, but not post AChR prepatterning, when these cells undergo a medial to lateral migration (Flanagan-Steet et al., 2005) (Figure 3C-E). Live imaging revealed that these Unplugged/MuSK-GFP puncta were highly dynamic and that they appeared to be actively maintained in the muscle center, as puncta that strayed from the center rapidly moved back to the central position (Movie S1).

To determine whether these puncta consisted of cytoplasmic protein aggregates or whether they represent a regulated pool of endocytosed protein, we co-injected *3.8unp:unplugged-RFP* with *rab7-GFP*, a late endosome marker, and *rab11-GFP*, a marker for exocytic/recycling endosomes (reviewed in Hutagalung and Novick, 2011). Rab7-positive vesicles were present throughout the entire length of individual adaxial muscle cells, and only a very small fraction of Rab7-positive vesicles colocalized with Unplugged/MuSK (Figure S2D). Conversely, Rab11-positive vesicles were restricted to
the cell center, and the majority colocalized with Unplugged/MuSK (Figure 3F-F'''). Thus, unplugged/MuSK expressed under its endogenous promoter localizes just before and during the time of AChR prepatternning to Rab11-positive vesicles at the center of adaxial muscle, precisely where AChRs accumulate.

**Unplugged/MuSK-GFP accumulation in endosomes depends on wnt11r/wnt4a**

*Rab11* is a member of the large family of small GTPases, and regulates both the exocytic biosynthetic and the recycling pathway (Chen et al., 1998; Ren et al., 1998; Satoh et al., 2005; Sonnichsen et al., 2000; Ullrich et al., 1996). To determine if Unplugged/MuSK localization to exocytic/endocytic vesicles depends on *rab11* function, we expressed a dominant negative, GDP-restricted *rab11*, *rab11*(S25N) (Ren et al., 1998) stochastically in adaxial muscle cells. As shown in Figure 4, disruption of *rab11* function resulted in Unplugged-RFP distribution throughout the entire cytoplasm of adaxial muscle cells, suggesting that Unplugged/MuSK protein localization depends on the *rab11*-mediated exocytic and/or recycling pathway (Figure 4A-4B, quantified in 4G). Importantly, Rab11 localization is unaffected in unplugged/MuSK mutant adaxial cells (Figure S2E).

We next asked whether the localization of Unplugged/MuSK to Rab11-positive vesicles is functionally important for *wnt11r/wnt4a*-induced synapse initiation. If so, we would make the following predictions. First, the disruption of *rab11*-mediated processes in adaxial muscle cells would affect AChR prepatternning. Indeed, when we expressed *rab11*(S25N)-myc in adjacent muscle cells, we observed a decrease in AChR clustering in these cells but not in the surrounding wild-type cells (n=3, Figure 4B-4B’). A second prediction is that in the absence of Wnt signals, the subcellular localization of Unplugged/MuSK to recycling endosomes would be affected. Analysis of *wnt11r*
mutant/wnt4a morphant embryos revealed a nearly complete relocalization of Unplugged/MuSK-GFP from Rab11-positive vesicles (Figure 4C-4C”) to the cell membrane (Figure 4D-4D”). These results demonstrate that Wnt signaling is required to relocalize Unplugged/MuSK-GFP from the cell membrane to Rab11-positive vesicles, and strongly suggest that Unplugged/MuSK-GFP and Rab11 double positive vesicles are not exocytic, but rather recycling endosomes. Combined, our data show that trafficking of Unplugged/MuSK from the cell membrane to Rab11-positive endosomes initiates AChR prepatternning in a wnt4a/wnt11r dependent manner.

**Functional interdependence of PCP core proteins with Unplugged/MuSK-GFP**

Given the striking subcellular localization of Unplugged/MuSK-GFP in response to Wnt signals, we next examined the localization of several components of the noncanonical Wnt signaling pathway. We previously used a dominant negative version of disheveled, dishevelled(DEP+), that specifically blocks noncanonical Wnt signaling (Axelrod et al., 1998; Heisenberg et al., 2000; Jing et al., 2009; Wallingford et al., 2000), to demonstrate that disheveled-dependent noncanonical signaling in adaxial muscle is critical for AChR clustering. Although it is known that Dishevelled can bind the juxtamembrane/kinase domain of the Unplugged/MuSK receptor (Luo et al., 2002), the subcellular localization of Dishevelled and other PCP core components during vertebrate neuromuscular synapse formation has not been established. To examine the subcellular localization of PCP core components, we expressed Daam1-GFP, Diversin/Ankrd6-YFP and Dishevelled-GFP in individual adaxial muscle cells and examined their localization just prior and during the period of AChR prepatternning.

Confocal analysis revealed that Daam1-GFP, Diversin/Ankrd6-YFP and Dishevelled-GFP localize to vesicle-like puncta that are strongly enriched in the center of
adaxial muscle fibers, identical to the localization of Unplugged/MuSK-GFP (Figure 5A-C, quantified in Figure S3A). Moreover, stochastic expression of a dominant negative version of daam1 (NDaam1) (Kida et al., 2007) and rhoA (rhoA(N19)) in adaxial muscle cells reduced AChR prepatternning and caused unplugged-like axonal guidance defects, confirming that PCP pathway components play a critical role in this process (Figure S3E-S3F and S3I-S3J'). Importantly, blocking the canonical Wnt pathway or the Wnt/Ca\(^{2+}\) pathway did not affect AChR clustering or axon guidance (Figure S3C-S3D and S3G-S3H'). Finally, coexpression of Dishevelled-GFP and Unplugged/MuSK-mKate revealed a high degree of colocalization between both proteins, strongly suggesting that in adaxial muscle cells, PCP core components such as Dishevelled associate with recycling endosomes (Figure 5E-5E’).

In Drosophila, PCP core components are dependent on one another for their correct subcellular localization. For example, loss of one PCP core component such as the frizzled receptor disrupts localization of other core proteins (reviewed in Wu and Mlodzik, 2009). The analogous prediction for this system would be that blocking the unplugged/MuSK receptor should interfere with Dishevelled-GFP localization, and vice versa. Remarkably, the subcellular localization of Dishevelled-GFP to the center of adaxial muscle cells is completely abolished in unplugged mutants, and instead Dishevelled-GFP is diffusely distributed throughout the cytoplasm (Figure 5C, 5D; quantified in 5H). Conversely, blocking dishevelled-dependent noncanonical signaling in adaxial muscle leads to a significant redistribution of Unplugged/MuSK-GFP from the endosomal compartment in the muscle center to a ubiquitous distribution at the membrane (Figure 5F-5G’), identical to what we observed when blocking wnt11r/wnt4a function (Figure 4F). Importantly, localization of Rab11-GFP is independent of dishevelled function, suggesting that rab11 likely functions ‘upstream’ of dishevelled, and
that the dependence of Unplugged/MuSK localization on *dishevelled* is highly specific (Figure S2E). Thus, several core PCP proteins colocalize with Unplugged/MuSK to presumptive endosomal vesicles in the center of adaxial muscle cells, precisely where the AChR prepattern forms. Moreover, Unplugged/MuSK and Dishevelled are dependent on one another for proper localization, consistent with the idea that both participate in a PCP-like signaling cascade to initiate synapse development.
Discussion

Initiation of neuromuscular synapses depends on multiple Wnt signals

Genetic mutants and knockouts have demonstrated that Wnts play a critical role in the assembly and plasticity of central synapses (Budnik and Salinas; Ciani and Salinas, 2005; Hall et al., 2000; Lucas and Salinas, 1997; Wu et al.; Yang et al.). Wnts are also involved at the invertebrate neuromuscular synapse (and reviewed in Davis and Ghosh, 2007; Klassen and Shen, 2007). However, the situation at the vertebrate neuromuscular junction is less well defined. Mice mutant for the frizzled co-receptor lrp4 lack all AChR prepatterning and mature NMJ synapses (Kim et al., 2008b; Weatherbee et al., 2006; Zhang et al., 2008), and knockdown of zebrafish wnt11r significantly reduces AChR prepatterning (Jing et al., 2009). While these reports strongly suggest that Wnts are critical for vertebrate neuromuscular synapse development, Wnt knockouts or genetic mutants with defects in neuromuscular synapse development have not yet been reported.

Here, we demonstrate that wnt11r null mutants display profound pre- and postsynaptic defects during the early phase of synapse development, without obvious defects in gross muscle development. Interestingly, we find that only the combined inactivation of wnt11r and wnt4a results in a complete loss of AChR prepatterning, almost identical to that observed in unplugged/MuSK mutants (Figure 1C and 1J). Wnt11r mRNA is expressed in the lateral mesoderm (Groves et al., 2005; Matsui et al., 2005) and (Figure S1G-S1H), consistent with a paracrine mode of action, while wnt4a is expressed in adaxial muscle, suggesting an autocrine mode of operation (Figure S1E-S1F). At the Drosophila neuromuscular junction, Wg and wnt5 function in a well-documented autocrine manner (Liebl et al., 2008; Packard et al., 2002) and future experiments are required to determine if in zebrafish adaxially-derived wnt4a indeed
activates the adaxially-expressed Unplugged/MuSK receptor directly. Combined, our results uncover the first genetic requirement for Wnts during vertebrate NMJ synapse development, and demonstrate that multiple Wnt signals initiate synapse formation.

**MuSK receptor localization to recycling endosome initiates synapse development**

Once thought to terminate transmembrane receptor signaling, the endocytic pathway is being increasingly appreciated for its ability to activate signaling of surface receptors such as the Notch and the EGF receptors (reviewed in Gagliardi et al., 2008), as well as chemokine receptors such as CXCR2. For example, it has been shown that in response to ligand stimulation, the CXCR2 receptor is internalized into Rab11-positive recycling endosomes, and that reducing CXCR2 recycling diminishes CXCR2 mediated chemotaxis (Fan et al., 2004). Similarly, Wnt mediated activation of Frizzled3 on axonal growth cones triggers Frizzled3 endocytosis and thereby initiates PCP signaling within growth cones (Shafer et al., 2011). This and other experiments have contributed to the current view that upon ligand binding, receptor endocytosis can lead to degradation, or to signaling, depending on the endocytic compartment to which the receptor is sorted (reviewed in Gagliardi et al., 2008). In the case of receptor tyrosine kinases, there is accumulating evidence that receptor endocytosis is required for signal transduction (Jing et al., 1992; Vieira et al., 1996; Yang et al., 2005).

Previous studies have shown that in response to ligand stimulation, MuSK undergoes rapid endocytosis in cultured myoblasts (Zhu et al., 2008). We find that in vivo and during the time of synapse initiation, Unplugged/MuSK is internalized from the cell membrane into Rab11-positive endosomes, and that this process is dependent on wnt11r and wnt4a. Moreover, rab11 function is important for proper Unplugged/MuSK protein localization and AChR pre patterning, demonstrating the importance of endosome
recycling to initiate *unplugged*/*MuSK* dependent processes (Figure 4). While we cannot exclude the possibility that *rab11* exerts its function directly on AChR trafficking, recycling of AChR receptors has been observed only at mature neuromuscular junctions, and only in response to muscle stimulation (Bruneau and Akaaboune, 2006; Martinez-Pena y Valenzuela et al., 2010), whereas at the stage of AChR prepatterning when we examined *rab11*-dependent endocytosis, neuromuscular junctions have not yet formed. Combined with the observations that *unplugged*/*MuSK* is critical for localization of Dishevelled to presumptive endosomes, and that Dishevelled function in muscle cells is critical for AChR prepatterning and axon guidance (see below and Jing et al., 2009), we favor the idea that *rab11* regulates primarily Unplugged/*MuSK* internalization.

Finally, we previously showed that forcing Unplugged/*MuSK* expression throughout the entire surface of muscle cells did not alter synapse initiation or position, suggesting the existence of additional regulators (Jing et al., 2009). Our findings that Unplugged/*MuSK* internalization to endosomes spatially restricts Unplugged/*MuSK* function and hence positions AChR clusters to the muscle center explain our previous results, and strongly suggest that Unplugged/*MuSK* internalization to spatially restricted endosomal compartment determines where along the muscle cell future synapses will form.

**Endocytosis, planar cell polarity and the initiation of neuromuscular synapses**

The internalization of Unplugged/*MuSK* to Rab11-positive endosomes and the requirement of *rab11*-mediated endocytosis to initiate AChR prepatterning suggests that the signaling proteins downstream of *unplugged*/*MuSK* might localize to and be active in Rab11-positive endosomes. In fact, we find that several core components of the PCP pathway, including Dishevelled, localize to the center of muscle cells, precisely where
AChR clusters accumulate, and we find that Dishevelled localization is unplugged/MuSK dependent (Figure 5C-5D). Conversely, blocking dishevelled function affects membrane-bound Unplugged/MuSK, preventing it from internalizing to the endosomal compartment, which ultimately results in defects in AChR prepatterning and motor axon guidance (Jing et al., 2009) and (Figure 5F-5G’’). This reciprocal functional dependency is intriguingly reminiscent of how the PCP pathway patterns Drosophila wing disc cells, albeit the precise mechanism initiating PCP signaling there is still unresolved (reviewed in Strutt and Strutt, 2009). Nonetheless, our results provide compelling evidence for a model in which Wnt ligands initiate a unplugged/MuSK-dependent signaling cascade in Rab11-positive recycling endosomes, where core PCP components modulate the cytoskeleton to determine the precise location of future synapses along the anterior-posterior axis of muscle cells (Figure 6).

Specifically, we propose a model (Figure 6) in which wnt11r and wnt4a trigger translocation of the Unplugged/MuSK receptor from the cell surface into Rab11-positive recycling endosomes. Importantly, these endosomes are enriched and actively maintained in the center of the muscle fiber. Once endocytosed, Unplugged/MuSK associates with Dishevelled (Luo et al., 2002), which is known to stimulate signaling following Wnt induced receptor endocytosis (Chen et al., 2003; Gao and Chen). For example, upon wnt11-mediated endocytosis of the Frizzled receptor, Dishevelled triggers noncanonical signals critical for Xenopus gastrulation (Kim et al., 2008a).

Downstream of dishevelled, the noncanonical Wnt pathway bifurcates into several branches, including into a daam1 dependent branch that mediates cytoskeletal reorganization (reviewed in Montcouquiol et al., 2006). We find that in addition to binding Unplugged/MuSK, Dishevelled also colocalizes with and binds to Diversin/Ankrd6 and Daam1, two proteins with well-characterized functions in PCP
signaling (Habas et al., 2001; Itoh et al., 2009; Kida et al., 2007; Moeller et al., 2006). *Daam1* is a member of the Formin family of proteins and is a central player in cytoskeletal reorganization (Alberts, 2002; Wallar and Alberts, 2003), in part through *rhoA* (Habas et al., 2001), a small GTPase known for its ability to modify the actin cytoskeleton (Maekawa et al., 1999; Matsui et al., 1996). Consistent with this view, we find that inhibiting *rhoA* function in adaxial muscle cells impairs AChR clustering (Figure S3), although it remains unclear how modifications of the actin cytoskeleton affect AChR clustering during synapse initiation *in vivo*. In cultured myotubes, actin dynamics have been shown to regulate AChR trafficking and accumulation (Dai et al., 2000; Lee et al., 2009; Pato et al., 2008). Regardless of the precise mechanism, our results demonstrate that core members of the PCP pathway known to regulate cytoskeleton dynamics colocalize with Unplugged/MuSK and play *in vivo* roles during synapse initiation.

One outstanding question is the mechanism by which Rab11/Unplugged/MuSK-positive endosomes localize to the center of muscle cells. Rab11 retains its subcellular localization even in the absence of unplugged/MuSK and *dishevelled* (Figure S2), which suggests that muscle cells have an inherent, unplugged/MuSK independent ‘polarity.’ Moreover, Rab7-positive late endosomes are distributed throughout muscle cells, excluding a ‘general’ endosome localization mechanism. The restricted localization of Rab11 is reminiscent of the situation in Drosophila sensory organ precursor cells (SOP), where Rab11 is localized to only one of two daughter cells following cell division, independent of the Par complex (Emery et al., 2005). Instead, Rab11 localization requires *nuclear fallout* (also known as Arfophilin, Eferin or Rab11-FIP3), which has been proposed to provide a link between *rab11* and the dynein light intermediate chain (Horgan et al., 2010). If this and other *rab11* effector proteins also play a role in the
localized accumulation of AChRs and ultimately synapse initiation remains an open question.

Overall, these data provide a conceptual link between how activation of the Unplugged/MuSK receptor on the surface of a muscle cell directs clustering of AChRs at the center of muscle cells. Finally, our results suggest a basic mechanism by which Wnt signals shape synapse localization through localized receptor endocytosis.
Material and Methods

**Whole Mount Immunocytochemistry and Microscopy**

Embryos were fixed and stained as described by (Zeller et al., 2002) and labeling of AChRs was achieved via the method described by (Jing et al., 2009). The following antibodies and dilutions were used: znp-1 (1:200, DSHB), myc (9E10, 1:1000, Covance), F59 (1:20, DSHB). Embryos were imaged with LCS (Leica) and IX81 (Olympus) confocal microscopes.

**Quantification of puncta, AChR clusters and RNA-injection membrane intensity**

For puncta and AChR counts, confocal images were projected into a single plane and converted to a 16 bit image using Metamorph. Puncta were counted using the “count nuclei” function, with the following parameters for each condition: AChRs imaged at 20X magnification, AChRs imaged at 60X magnification and endocytosed puncta imaged at 60X magnification with minimum/maximum lengths of 5/30, 5/100 and 1/10 respectively and minimum average intensities of 30, 50 and 100 respectively. The results were imported to Microsoft Excel and Graphpad Prism for statistical analysis and plotting. Single confocal slices of images of RNA-injected embryos were converted to 16 bits using Metamorph, and the maximum pixel intensity at three membranes per image was manually recorded for both color channels.

**Whole-mount in situ hybridization**

Colorimetric in situ hybridizations were performed as described in (Schneider and Granato, 2006) with the previously published wnt4a in situ probe ‘eu648’ (Thisse, 2005) and the previously published full-length wnt11r probe (Jing et al., 2009).
Plasmid Construction

*Rab11a*-GFP and *rab11*(S25N) in PCS2+ (Clarke et al., 2006) were gifts from Clare Buckley (Kings College London) and Gwyn Gould (University of Glasgow). *mDiversin* in eYFP C1 was a gift from Tobias Schäfer (University Hospital Freiburg) (Haribaskar et al., 2009). *Daam1*-GFP in PCS2+ was a gift from Raymond Habas (Temple University, Philadelphia) (Liu et al., 2008). *XDishevelled*-GFP in PCS2+ was a gift from Peter Klein (University of Pennsylvania), *dishevelled*(DEP+) was cloned as previously described (Jing et al.) and *dishevelled*(DIX) was subcloned from the full length construct using the following primers:

AAAAGAATTCGCCACCATGGCGGAGACTAAAGTGATTTAC (forward)
AAAAGAATTTTCTAGAGCTCTCTGACCTCTGTAGCAGF (reverse).

*NDaam1*-HA in pcDNA3 was a gift from Yasuyuki Kida (Tohoku University, Japan) (Kida et al., 2007). *CamKII*(K42M) and *rhoA*(N19) in PCS2+ were gifts from Juan Carlos Izpisua Belmonte (Salk Institute, CA, USA) (Matsui et al., 2005). *Unplugged*, *unplugged*ΔCRD (Jing et al., 2009) and *wnt11r* were amplified from genomic DNA and cloned into the PCS2+ vector. Once cloned into PCS2+ (or in the case of *diversin*-YFP and *daam1b* in eYFP C1 and pcDNA3 vectors respectively), the constructs were tagged with either GFP or 5x-Myc using standard cloning procedures. Fusion constructs were PCR amplified from PCS2+ and cloned into pENTR/D-TOPO (Invitrogen, USA) using the following primers:

5’ CACCCAAGCTACTTGTCTTTTTTG 3’ (forward)
5’ GGTACCGGGCCCAATGCATT 3’ (reverse).

Constructs were then transferred to a homemade Gateway destination vector containing either the 3.8*unp* promoter (below) or the *smyhc1* promoter using the Gateway LR Clonase II enzyme mix (Invitrogen, USA). The 3.8*unp* promoter, which includes the
5'UTR and first intron of *unplugged*, was amplified from Fosmid CH1073-179D18 (BACPAC Resources, Oakland, CA, USA) using primers 5' TGGTCTTTTCGTGGAGTTCC 3' (forward) and 5' TGGTTGTGAGAAGATAAAGATCTG 3' (reverse).

**Chimeric embryos**

Cell transplants were performed as described in (Zeller and Granato, 1999) with the modification that embryos were fixed at 20 somites, permeabilized for 5 minutes with 0.1% collagenase, stained for 1 hour with fluorescently conjugated bungarotoxin and mounted in vectashield for confocal imaging.

**DNA, RNA and Morpholino Injections**

Destination vectors containing either *GFP* or *myc* tagged constructs were microinjected into one cell-stage embryos as previously described (Thermes et al., 2002). *Wnt11r, unplugged*-GFP and *unplugged*-ΔCRD-GFP mRNA were *in vitro* transcribed from Not1 linearized PCS2+ expression vectors using SP6 mMessage mMachine Kit (Ambion, USA), diluted in 0.1M KCl and microinjected into embryos at the one cell-stage. 10ng of a previously published *wnt4a* translation blocking morpholino with the sequence (CTCCGATGACATCTTTAGTGGAATC) was injected at the one-cell stage (Matsui et al., 2005).
Figures and Legends

Figure 3.1 Loss of wnt11r and wnt4a phenocopies unplugged mutant

(A-K) Lateral view of trunk (top, dorsal; left, anterior) stained for AChR clusters (bungarotoxin, red) and motor axons (znp-1, green) in 20 somite and 26 hpf embryos.

(A, B) Three hemisegments in a wild-type embryo (hemisegment boundaries marked with dotted lines) with prepatterned AChR clusters along the center of each hemisegment (cluster width marked by bracket) (A) and three wild-type hemisgments in which motor axons have made synaptic contacts along the length of the trunk (B).

(C, D) Three hemisegments in an unplugged mutant showing a complete dispersion of AChR clusters (C) and axon guidance errors in 26 hpf unplugged mutants (arrow points to axon branch) which occur in 85% of axons (D).

(E-G) A single hemisegment from a wnt11r embryo showing an expanded AChR prepattern (width bracketed, 61% occurrence) (E), and a single hemisegment showing a complete loss of AChR prepattern (39% of occurrence; F). Three hemisegments from a 26 hpf wnt11r mutant showing unplugged-like axon branching (arrow) in 26% of axons (G).

(H, I) Three hemisegments from a wild-type embryo injected with 10ng of wnt4a morpholino showing wild-type-like prepatterning (H) and wild-type-like axons with only 6% guidance errors (I).

(J, K) Three hemisegments from a wnt11r mutant injected with 10ng of wnt4a morpholino showing unplugged-like AChR dispersal in all hemisgements (J) and guidance errors in 70% of axons (K).

(L) Quantification of increase in AChR prepattern width in wnt11r mutants as compared to wild-type (p< 0.01 Student’s two-tailed t-test, unequal variance).

Scale bar = 10 micrometers.
Figure 3.2 Addition of \textit{wnt11r} triggers a loss of Unplugged/MuSK-GFP at the membrane in a CRD-dependent manner.

(A-D) 4.5 hpf dome-stage embryos expressing mCherry-CAAX membrane marker (red) and either Unp-GFP or UnpΔCRD-GFP (green) in separate and merged channel views.

(A-A") Wild-type embryos showing strong Unp-GFP on the membrane.

(B-B") Wild-type embryos injected with \textit{wnt11r} mRNA with reduced Unp-GFP at the membrane (white bracket highlights one area of reduced GFP signal).

(C-C") Wild-type embryos showing strong UnpΔCRD-GFP expression at the membrane, which is not reduced in the presence of \textit{wnt11r} mRNA (D-D").

(E) Quantification of effect of \textit{wnt11r} on Unp-GFP membrane localization (p= 4.6 E-05 Student’s two-tailed t-test, unequal variance).

(F) Quantification of effect of \textit{wnt11r} on UnpΔCRD-GFP membrane localization (p= 0.39 Student’s two-tailed t-test, unequal variance).

Scale bar = 10 micrometers.
Figure 3.3 Unplugged/MuSK-GFP localizes to Rab11-positive endosomes in the center of presynaptic muscle fibers.

(A) Mosaic expression of Unp-GFP driven by the smyhc1 promoter in two slow muscle cells (green) with prepatterned AChR clusters (bungarotoxin, red) along the center of the cells.

(B) 3.8unp:GFP (green) and membrane marker smyhc1:mCherry-CAAX (red).

(C-E) 3.8unp:unp-GFP (green) and smyhc1:mCherry-CAAX (red) in a ‘young’ cell that does not yet have prepatterned AChR clusters (C), and in an ‘older’ cell in which prepatterned AChR clusters have been replaced by synaptic clusters (accumulation of protein at the myoseptal boundary is marked with an arrow) (E).

(D) 3.8unp:unp-GFP in a slow muscle cell (green) with AChRs (bungaroxin, red) showing that Unp-GFP puncta aggregate beneath the overlying prepatterned AChR clusters.

(F-F'') A single confocal slice showing colocalization of Unp-RFP (red) and early/recycling endosome marker Rab11-GFP (green) expressed under the 3.8unp promoter in a normal view (F) and zoomed-in view (F'').

Scale bar = 10 micrometers.

See also Figure S2 and Movie S1.
Figure 3.4 Unplugged/MuSK’s punctate localization is disrupted in the absence of *rab11* and *wnt11r/4a*

(A-A’) Coexpression of *smyhc1:rab11*(S25N)-GFP (green) and 3.8*unp:unp-RFP* (red; red channel alone in A’), demonstrating disruption of Unp-RFP’s endosomal localization.

(B-B’) *smyhc1:rab11*(S25N)-GFP (green) expressed in two adjacent muscle cells showing disruption of AChR clusters (bungarotoxin, red) at the interface of the two mosaic fibers but not in an adjacent wild-type cell (arrow).

(C-C’’) 3.8*unp:unp-GFP* (green) in a wild-type embryo with normal protein distribution (C) and a zoomed-in view of dotted white box showing very little protein at the membrane (C’-C’’).

(D-D’’) 3.8*unp:unp-GFP* (green) in a *wnt11r* mutant/ *wnt4a* morphant (D), and zoomed-in view of dotted white box showing accumulation of protein at the membrane (D’-D’’).

(E-F) Quantification of central enrichment of Unp-RFP puncta when coexpressed with Rab11(S25N)-GFP (E) (p values for muscle subdivisions 1-5 = 0.6, 0.27, 0.01, 0.38, 0.34 respectively), or when expressed in a *wnt11r* mutant/*wnt4a* morphant background (F; p values for muscle subdivision 1-5= 0.87, 0.12, 0.04, 0.36, 0.97 respectively).

Scale bar = 10 micrometers A-D, 1 micrometer for C’-D’’
Figure 3.5 Several noncanonical PCP proteins localize to centralized puncta in the presence of unplugged/MuSK.

(A-B) Daam1-GFP (green) (A) and Diversin-YFP (green) under the 3.8unp promoter (B) localize to centrally-enriched puncta in muscle cells coexpressing mCherry-CAAX under the smyhc1 promoter (red).

(C) Dsh-GFP under the 3.8unp promoter (green) localizes to centrally-enriched puncta in fibers coexpressing mCherry-CAAX (red).

(D) The punctate localization of Dsh-GFP is lost in unplugged mutant muscle cells.

(E-E”) Zoomed-in view of center of muscle cell showing colocalization of Dsh-GFP (green) and Unp-mKate (red) under the 3.8unp promoter.

(F-F’’) Unp-GFP under the 3.8unp promoter (green) localizes to centrally-enriched puncta in muscle cells coexpressing mCherry-CAAX (red) in zoomed-out (F) and zoomed-in views of the region marked with dotted white box (F’-F’’).

(G-G’’) Unp-GFP remains on the membrane of muscle cells coexpressing Dsh(DEP+)-Myc; zoomed-out (G) and zoomed-in views of the region marked with dotted white line (G’-G’’).

(H) Quantification of reduction in centrally-localized Dsh-GFP puncta in unplugged mutants (p values for muscle subdivision 1-5: 0.49, 0.09, 0.05, 0.04, 0.02 respectively).

See also Figure S3.
**Figure 3.6 Model for Wnt dependent receptor endocytosis directing synapse position.**

(A) Schematic of a somitic hemisegment with parallel early muscle cells in grey. AChRs (red) are clustered in the center of the muscle fibers along the path where the motor axon (green) will extend.

(B) Zoomed-in view of dotted black box in (A) showing a cross-section of a single wild-type muscle fiber. Motor axon growth cones (green) are navigating in the center of cells, precisely where AChRs (red) are clustered. Upon Wnt11r and Wnt4a (yellow) exposure, Unplugged/MuSK (blue) translocates from the membrane into Rab11-positive endosomes restricted to the center of muscle cells. The cytoplasmic domain of Unplugged/MuSK binds Dishevelled (orange), and nucleates accumulation of Diversin/Ankrd6 (brown), Daam1 (purple), and RhoA (green) to modify the cytoskeleton, ultimately anchoring AChRs to the cell center.

(C) Zoomed-in view of showing a cross-section of a wnt11r mutant/wnt4a morphant muscle cell, in which Unplugged/MuSK, Dishevelled, Daam1, Diversin and RhoA are no longer localized in the center. Consequently, AChRs and growth cones (green) are no longer restricted to the muscle center.
Supplemental Figure 3.1 *wnt4a* and *wnt11r* are expressed in the vicinity of adaxial muscle cells and loss of Wnts does not affect muscle development

(A-D) Muscle fibers (F59, red) show normal morphology in wild-type embryos (A), *wnt11r* mutants (B), *wnt4a* morphants (C) and *wnt11r* mutant/*wnt4a* morphants (D) although the axons in (B) and (D) are affected.

(E-H) Cross-sections of 20 somite and 26 hpf embryos stained with a DIG-labeled RNA probe for *wnt4a*, showing strong labeling in the spinal cord (dotted white line) as well as more diffuse staining throughout the somites (A-B). Staining with a *wnt11r* probe revealed strong labeling in the spinal cord and adjacent somites (C-D). Notochord marked with (*).
Supplemental Figure 3.2 3.8unp:unp-GFP rescues AChR clustering at levels to the same degree as transplanting wild-type cells into unplugged mutants and distribution of Rab11-GFP and Rab7-GFP in muscle cells

(A-A') Control expression of 3.8unp:GFP (green) in unplugged mutants fails to rescue AChR prepatternning (bungarotoxin, red).

(B-B') Expression of 3.8unp:unp-GFP in unplugged mutants rescues AChR clustering cell-autonomously in 47% of embryos (n= 22).

(C-C') Transplantation of wild-type fibers labeled with rhodamine dextran into unplugged mutants rescues prepatternning in 57% of embryos (n=40).

(D-D'') Weak colocalization of of Rab7-GFP and Unp-mKate under the 3.8unp promoter.

(E) Distribution of Rab11-GFP puncta in wild-type, in unplugged mutants or in cells coexpressing Dsh(DEP+)-myc.
Supplemental Figure 3.3 Expression of dominant negative PCP but not of canonical Wnt pathways components in muscle cells disrupts AChR clustering and axon guidance

(A) Distribution of puncta of various proteins under the control of the 3.8unp promoter among five laterally delineated segments of muscle cells, normalized to 100.

(B-F) Stochastic expression of smyhc1:GFP (B), smyhc1:dsh(DIX)-myc (C), smyhc1:camKII(K42M)-myc (D) in muscle cells just dorsal to the horizontal myoseptum in 27 hpf embryos does not induce axon guidance errors. Expression of smyhc1:NDaam1-myc (E) and smyhc1:rhoA(N19)-myc (F) does induce guidance errors (white arrow, motor axons stained with znpl-1 in green).

(G-J') Stochastic expression of smyhc1:Ndaam1-myc (I-I') and smyhc1:rhoA(N19)-myc (J-J') but not smyhc1:GFP (G-G') or smyhc1:dsh(DIX)-myc (H-H') in 20 somite embryos causes a reduction in AChR prepatterned clustering (bungarotoxin, red) at the interface between the two expressing fibers but not in adjacent wild-type fibers.

Scale bar =10 micrometers.
CHAPTER 4
DISCUSSION

Since the identification of *unplugged/MuSK* as a critical regulator of AChR clustering and axon guidance in the developing embryo, the laboratory has been pursuing a mechanistic link to explain how a receptor that is strictly localized to muscle fibers might non-autonomously affect axons migrating over the surface of those fibers. This question is made all the more complex by the migratory nature of the muscle fibers expressing *unplugged/MuSK*. *Unplugged/MuSK*-expressing slow-twitch fibers initially pave the path traversed by motor axons but migrate away prior to the arrival of the growth cone, while only the muscle pioneers remain at the horizontal myoseptum. The migrating slow-twitch muscle fibers are replaced by fast-twitch muscle fibers, which do not express *unplugged/MuSK*. The newly positioned fast-twitch muscle fibers and the remaining slow-twitch muscle pioneers then make synaptic contacts with the migrating axon.

Several of these fast-twitch muscle fibers appear to pre-cluster AChRs prior to the arrival of the nerve in a manner similar to slow-twitch muscle fibers (Gordon, unpublished data). This observation is enigmatic given that fast-twitch fibers lack *unplugged/MuSK* expression at this early stage. However, upon closer analysis, I observed that what appeared to be AChR prepatterned clusters on the fast-twitch muscle fibers were actually clusters on long, membranous extensions of migrating slow-twitch muscle fibers that stretched the entire distance from the axonal path to the migrating cell body (Gordon, unpublished data). I am unsure how long these membranous extensions persist or over what distance the slow-twitch muscle fibers are capable of stretching, but I suspect that they are absent by the time the slow-twitch
muscle fibers complete their lateral migration and growth cones form synaptic contacts with fast-twitch fibers.

Although these membranous extensions explain the appearance of an occasional prepatterned AChR cluster amidst fast-twitch muscle fibers, they do not explain how molecular changes in the slow-twitch muscle fiber can affect the motor nerve without physical contact between the two cells. Because of this gap in our understanding, my model necessarily invokes an indirect interaction between muscle fibers and motor axons, with slow-twitch muscle fibers inducing relatively stable alterations in the extracellular matrix lining the future path of motor axons.

One piece of evidence in support of this model is the observation that accumulation of two repulsive guidance cues, Tenascin-C and Chondroitin Sulfate Proteoglycan, in the extracellular space overlying slow-twitch muscle fibers is reduced in the absence of unplugged/MuSK (Schweitzer et al., 2005; Zhang and Granato, 2000). However, global inhibition of Chondroitin synthesis does not induce unplugged-like axon guidance errors (Zhang et al., 2004), suggesting either that Chondroitin is not the key component responsible for guiding motor axons or that other components such as Tenascin are compensating in its absence. Consequently, it would be interesting to identify additional ECM components that are deposited by slow-twitch muscle fibers in the presence of unplugged/MuSK.

To further explore the role of Tenascin, I constructed a fluorescently tagged version of Tenascin and plan to express it in slow-twitch muscle fibers and document its localization in the presence and absence of unplugged/MuSK. The observation that two repulsive guidance cues are affected by unplugged/MuSK suggests that the readouts of unplugged/MuSK signaling are primarily inhibitory. However, this does not rule out the possibility that positive cues may be influenced by unplugged/MuSK as well.
In addition to answering the question of how slow-twitch muscle fibers indirectly communicate with migrating motor axons, I wanted to explore how activation of a surface receptor could translate into the spatial restriction of clustered AChRs in the center of the muscle fiber. Paradoxically, overexpression of unplugged/MuSK, which results in unplugged/MuSK accumulation throughout the entire anterior-posterior axis of the muscle membrane, is capable of inducing central AChR clustering. This demonstrates that the localization of the receptor, at least at the membrane, is not critical for its proper function. I therefore hypothesized that another factor, either a Wnt ligand or another component in the muscle fiber, is ultimately responsible for spatial restriction of signaling. Central presentation of a ligand to a ubiquitously expressed receptor might activate only centrally-localized receptors. Likewise, central accumulation of a membrane-bound or cytoplasmic unplugged/MuSK binding partner might allow only those receptors in the center to signal.

To begin testing the involvement of Wnts in the spatial restriction of unplugged/MuSK signaling, I examined the localization of Wnt11r protein in a wild-type embryo. I observed that Wnt11r protein accumulates more heavily in the extracellular space adjacent to the center of the muscle fiber than at either end. Moreover, this central accumulation was lost in unplugged mutants. However, I was not able to determine from this assay whether the enrichment of Wnt11r triggered centralized activation of the unplugged/MuSK receptor or whether Wnt11r accumulated centrally by binding to an already localized cue. I thought it unlikely that a long-range diffusible ligand such as Wnt could be presented in such a spatially restricted manner, and favored the hypothesis that Wnt was becoming trapped in the central ECM by binding to an already localized factor. My findings that unplugged/MuSK localizes to endosomes in the center of the muscle fiber and that these endosomes maintain a central localization
even in the absence of unplugged/MuSK argue that an independent, muscle specific factor is responsible for limiting recycling machinery to the center of the fiber, which then forms the basis for other central specializations. These findings suggest that an alternate, unplugged/MuSK-dependent cue is necessary for the initial polarization of the muscle fiber, though it does not rule out the possibility that unplugged/MuSK and Wnt contribute to the maintenance and/or stabilization of the central muscle zone.

One candidate factor for the master central zone organizer is LRP4, which is known to bind to unplugged/MuSK (Kim et al., 2008b). In LRP4 mutant mice, all centralized AChR prepatterning is abolished, just as in MuSK mutants (Weatherbee et al., 2006). It would be interesting to examine the localization of the endocytic marker Rab11 in LRP4 mutants to determine whether LRP4 is indeed the factor that controls the localization of recycling machinery.

An alternative hypothesis is that the central accumulation of synaptic components and recycling machinery is initially due to a transcription/translation bias from the centrally positioned nucleus. This argument has been proposed to explain the central activation of unplugged/MuSK in polynucleated muscle fibers, where transcription is triggered earlier in the central, synaptic nuclei than the peripheral nuclei (Burden, 2002). Whether this model applies to fibers in which there is a single, centrally localized nucleus remains to be seen. Regardless, some other factor would still be necessary to stabilize these central components, as some proteins, including late endosome markers, are produced by the same nucleus and are not centrally enriched. One potentially informative experiment would be to alter the localization of the nucleus by disrupting nuclear anchoring proteins and then test whether this perturbation affects centralized AChR clustering.
The data presented in this thesis support a model in which *unplugged*/MuSK signaling from endosomes is required for central accumulation of AChR receptors. This argues that the endocytosed receptor, rather than the membrane-bound receptor, is critical for establishing the central zone of the fiber. To further address this question, I am planning to inject a construct coding for a fusion between Rab11, which is localized to endosomes, and the cytoplasmic domain of Unplugged/MuSK-GFP into *unplugged* mutant muscle fibers. Following injection, I will test whether the fusion protein is capable of rescuing AChR clustering. If the cytoplasmic domain, which is restricted to endosomes by virtue of the fusion to Rab11, is capable of rescuing the *unplugged* mutant, I can conclude that endosomal, rather than membrane localization is critical for *unplugged*/MuSK signaling.

Another outstanding question is how *unplugged*/MuSK affects AChR clustering once it is localized to central endosomes. I propose a model in which Unplugged/MuSK recruits several planar cell polarity proteins to its central location, which then alter the cytoskeleton. These local cytoskeletal manipulations may then affect AChRs, which are known to rely on cytoskeletal integrity for proper clustering (Lee et al., 2009; Pato et al., 2008). However, I currently have little evidence linking the cytoskeleton to this process. Other lab members are gathering preliminary data to test for cytoskeletal involvement by imaging microtubule dynamics in wild-type and *unplugged* mutant muscle fibers.

In this thesis work, I identified several downstream signaling components that might form a bridge between *unplugged*/MuSK and the cytoskeleton. Specifically, I discovered a role for the planar cell polarity components Daam1 and Diversin/Ankrd6 in AChR clustering and axon pathfinding, but both of these components are known to primarily complex with Dishevelled/Prickle on the distal side of *Drosophila* wing disk cells. I was not able to identify a clear role for one of the PCP components traditionally
localized to the proximal side of the wing disk such as Vangl2 and Prickle (reviewed in Strutt, 2003).

In my exploration of Vangl2, I found that expression of a dominant negative version of Vangl2 (Vangl2 delta PDZ) in slow-twitch muscle fibers induced dramatic prepatternning and axon guidance defects. I next attempted to analyze the Vangl2 zebrafish mutant, but it exhibited too many non-specific defects (due to impaired early embryonic convergent-extension) to accurately analyze AChR prepattern disruption. To overcome this barrier, I transplanted mutant muscle fibers into a wild-type host, but did not see any increase in axon guidance or AChR clustering errors in the hemisegments containing mutant fibers, which conflicted with what I observed using the dominant negative Vangl2 construct.

Zebrafish have two versions of Vangl, Vangl1 and Vangl2, so I hypothesized that the dominant negative Vangl2 was affecting both versions, and that Vangl1 might be compensating in the absence of Vangl2 and vice versa. Consistent with this, a published translation-blocking morpholino against Vangl1 (Borovina et al., 2010) did not induce any axon guidance or AChR defects when injected at an optimal dose. To test whether the two versions of Vangl compensate for one another, morpholino-injected, mutant muscle fibers would need to be transplanted into a wild-type host and analyzed for AChR and axon guidance defects. However, when expressed under the unplugged/MuSK promoter, Vangl2 is diffusely localized throughout the cytoplasm rather than clustered in puncta like Daam1 and Diversin/Ankrd6, which suggests that it may not be involved in physically establishing the central zone of the muscle fiber.

Additionally, I examined the localization and function of Prickle, which, like Vangl, has multiple homologues in zebrafish: Prickle1a, Prickle1b, Prickle2 and Prickle3. I obtained Prickle1b mutants, but did not observe any unplugged-like defects. However,
injection of either a translation blocking or a splice blocking morpholino against Prickle1a induced strong AChR prepattern and axon guidance defects. Expression of Prickle-GFP in slow-twitch muscle fibers resulted in accumulation of the protein in large puncta at the dorsal and ventral membranes of the fiber. These Prickle-GFP puncta do not appear to be localized to endocytic vesicles as is the case with the other planar cell polarity proteins I identified in this thesis work. The unusual localization may be consistent with the documented exclusion of Prickle from Dishevelled/Diversin complexes (reviewed in Veeman et al., 2003), but it is unclear what role, if any, Prickle is playing in the unplugged/MuSK signaling pathway or how its localization fits into my working model.

One final protein that I have not yet examined for its involvement in the unplugged/MuSK signaling pathway is Frizzled. It is possible that Frizzled, a known Wnt receptor and a core member of the planar cell polarity pathway, is acting as a co-receptor for Wnt11r/4a along with Unplugged/MuSK. Alternatively, it is possible that unplugged/MuSK is the lone receptor and is solely responsible for triggering downstream signaling events that might attributed to Frizzled in other tissues. It would be informative to examine the localization of fluorescently tagged Frizzled in slow-twitch muscle fibers in the presence and absence of unplugged/MuSK as a starting point for this line of inquiry.

Together, these observations support a model in which Wnt11r and Wnt4a are uniformly presented to slow-twitch muscle fibers, which are positioned along the future synaptic path, adjacent to the notochord. Wnt11r and Wnt4a then induce activation of Unplugged/MuSK, which is localized throughout the membrane of the muscle fiber, by promoting dimerization and auto-phosphorylation. Following activation, surface Unplugged/MuSK is endocytosed and shuttled to recycling endosomes, which are enriched in the center of the muscle fiber in an unplugged-independent manner.
Identifying the cue that is responsible for initially organizing these recycling endosomes is a critical question that merits further exploration.

Once in recycling endosomes, Unplugged/MuSK forms a stable complex with the PCP components Dishevelled, Daam1 and Diversin/Ankrd6. In the absence of unplugged/MuSK, Dishevelled, and likely Daam1 and Diversin/Ankrd6, are free-floating in the cytoplasm. These PCP components, along with RhoA, then may make alterations to the local cytoskeleton, which could affect either insertion or stabilization of centrally-localized AChRs. These cytoskeletal modifications may also direct the secretion of the inhibitory guidance cues Tenascin and Chondroitin Sulfate Proteoglycan in the extracellular space above the center of the muscle fiber. Presumably, other factors that bind and stabilize Wnt are also secreted in the central zone. The slow-twitch muscle fibers then migrate laterally and are replaced by fast-twitch fibers. Some slow twitch fibers maintain long membranous extensions that continue to cluster prepatterned AChRs, but these extensions are likely retracted by the time the slow-twitch fibers complete their migration.

When motor axons begin their migration, they encounter guidance cues that were deposited in the ECM by the slow-twitch fibers, and they are able to make properly positioned synaptic contacts with fast-twitch fibers. Altogether, these data provide a highly sought-after mechanistic link between receptor activation and the resulting fundamental changes to pre- and postsynaptic cells, while also providing an intriguing example of how endocytosis can be a means of enhancing, rather than quenching, receptor signaling.
BIBLIOGRAPHY


