Wnts contribute to neuromuscular junction formation through distinct signaling pathways

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**Key words**: Neuromuscular junction, β-catenin signaling, Planar cell polarity, Wnt, Vangl2.
Summary Statement

In this article, we provide evidence that the coordinated action of Wnt4 and Wnt11 contributes to mammalian neuromuscular junction (NMJ) formation through the activation of canonical Wnt signaling and the Wnt/Vangl2-dependent core Planar Cell Polarity pathways.
Abstract

Understanding the developmental steps shaping the formation of the neuromuscular junction (NMJ) connecting motoneurons to skeletal muscle fibers, is critical. Wnt morphogens are key players in the formation of this specialized peripheral synapse. Yet, the individual and collaborative functions of Wnts as well as their downstream pathways remain poorly understood at the NMJ. Here, we demonstrate through Wnt4 and Wnt11 gain of function studies in culture or in mice that Wnts enhance acetylcholine receptor (AChR) clustering and motor axon outgrowth. In contrast, loss of Wnt11 or Wnt-dependent signaling in vivo decreases AChR clustering and motor nerve terminal branching. Both Wnt4 and Wnt11 stimulate AChR clustering and mRNA downstream activation of the β-catenin pathway. Strikingly, Wnt4 and Wnt11 co-immunoprecipitate with Vangl2, a core component of the Planar Cell Polarity (PCP) pathway, which accumulates at embryonic NMJ. Moreover, mice bearing a Vangl2 loss of function mutation (looptail) exhibit a decreased number of AChR clusters and overgrowth of motor axons bypassing AChR clusters. Taken together, our results provide genetic and biochemical evidences that Wnt4 and Wnt11 cooperatively contribute to mammalian NMJ formation through activation of both the canonical and Vangl2-dependent core PCP pathways.
Introduction

Formation of the vertebrate neuromuscular junction (NMJ), a peripheral cholinergic synapse between motor neurons and skeletal muscle fibers relies on the accurate recognition and apposition of presynaptic motoneurons on postsynaptic muscle targets, a process achieved by a variety of organizing signals from both partners (Tintignac et al., 2015). Growing evidence in several vertebrate species, using both in vitro and in vivo models suggests that Wnt morphogens act as regulators of NMJ initiation and/or formation (Gordon et al., 2012; Henriquez et al., 2008; Jing et al., 2009; Messéant et al., 2015; Packard et al., 2002; Strochlic et al., 2012; Zhang et al., 2012). Yet Wnt function and the molecular mechanisms through which Wnts collaborate at the mammalian NMJ remain elusive and controversial.

Wnts are known to activate a canonical signaling pathway that is β-catenin (Ctnnb1)-dependent as well as several non-canonical ones such as that of the core planar cell polarity (PCP) pathway (Nusse, 2012). At the vertebrate NMJ, Wnt ligands transduce their signals through the activation of the receptor complex formed by the muscle specific tyrosine kinase MuSK and the low-density lipoprotein receptor-related protein4 Lrp4 and through the activation of classical Frizzled (Fzd) receptors (Avilés et al., 2014; Zhang et al., 2012; Strochlic et al., 2012; Gordon et al., 2012; Messéant et al., 2015). The MuSK/Lrp4 complex constitutes the central scaffold for the formation of the neuromuscular synapse (DeChiara et al., 1996; Kim et al., 2008; Weatherbee et al., 2006; Zhang et al., 2008). Activation of this complex is required for i) the early, nerve-independent muscle prepatterning, characterized by AChR aggregation in the prospective synaptic region of the muscle surface that helps to guide growing motor axons towards their final target, and ii) the late, nerve-dependent, differentiation and maturation of the synapse (Tintignac et al., 2015). This later step is orchestrated by the release of a nerve secreted isoform of agrin which binds to muscle Lrp4
leading to activation of MuSK and AChR clustering in the postsynaptic membrane (Kim et al., 2008; Zhang et al., 2008; Zhang et al., 2011; Zong et al., 2012).

Among the 19 Wnts currently identified in mammals, Wnt2, 3a, 4, 6, 7b, 9a and 11 directly interact with MuSK but only Wnt4, 9a and 11, enhance AChR clustering in muscle cells (Barik et al., 2014; Strochlic et al., 2012; Zhang et al., 2012). In zebrafish, both Wnt4a and Wnt11r initiate muscle prepatterning likely by stimulating PCP-dependent MuSK endocytosis in muscle cells (Gordon et al., 2012; Jing et al., 2009). In mice, although recent data have challenged the role of Wnts at the NMJ (Remédio et al., 2016; Discussed below), we have demonstrated that Wnt4 contributes to muscle prepatterning (Strochlic et al., 2012). Wnt signaling is also required for later steps of vertebrate NMJ differentiation. For example, Dishevelled (Dvl)1, a hub for Wnt signaling, interacts with MuSK and plays several roles during NMJ formation (Henriquez et al., 2008; Jing et al., 2009; Luo et al., 2002; Wang et al., 2014). Wnt3 expressed by motoneurons enhances AChR clustering in developing chicken wings and agrin-induced AChR clustering in cultured myotubes through a non-canonical signaling pathway (Henriquez et al., 2008). In contrast, Wnt3a disperses agrin-induced AChR clusters by down-regulating rapsyn expression in a β-catenin dependent manner in muscle cell culture (Wang et al., 2008). In addition, muscle β-catenin gain- or loss-of-function in mice, revealed its role in pre- and postsynaptic differentiation consistent with a critical level of β-catenin expression required for the proper formation of the NMJ (Li et al., 2008; Liu et al., 2012; Wang and Luo, 2008; Wu et al., 2012a; Wu et al., 2015).

Here we have used a set of mutant mice, as well as newly designed in vivo tools and biochemical assays, to identify the signaling pathways activated by Wnt/receptor interaction and their function in pre and postsynaptic differentiation of mammalian NMJs. We show that Wnt11 cooperates with Wnt4 to enhance AChR subunit mRNA levels and aneural AChR clustering in cultured muscle cells in part through activation of β-catenin signaling. In
addition, in vivo application of both Wnt4 and Wnt11 before NMJs begin to form, enhances AChR clustering and motor axon outgrowth. In contrast, lack of Wnt11 or inhibition of all Wnt-dependent signaling in vivo decreases AChR clustering and nerve terminal arborization. Specific inhibition of the Wnt canonical pathway similarly affects AChR distribution but not axonal branching suggesting that distinct branches of Wnt signaling regulate nerve terminal arborization. Interestingly a significant number of axons fail to terminate at AChR clusters and grow exuberantly beyond the prepatterned region of the muscle. Finally, we show that: i) both Wnt11 and Wnt4 co-immunoprecipitate with Vangl2, a key component of the core PCP pathway, ii) Vangl2 accumulates at embryonic NMJs and iii) mice bearing the Vangl2 loss of function mutation (Looptail; Vangl2^{Lp/Lp}) exhibit disrupted AChR clusters and axons outgrowth that bypass AChR clusters. Taken together, our results provide compelling evidence that the coordinate action of Wnt4 and Wnt11 regulate NMJ formation through activation of both the canonical and Vangl2-dependant PCP pathways.
Results

Wnt4 and Wnt11 cooperatively enhance AChR clustering.

In view of the complex and still-debated role of Wnt4 and Wnt11 in vertebrate NMJ formation (Gordon et al., 2012; Jing et al., 2009; Messéant et al., 2015; Remédio et al., 2016; Strochlic et al., 2012), we decided to investigate events downstream of Wnt4 and Wnt11 and whether they cooperate to induce AChR clustering. Wnt11 alone is known to enhance AChR clustering in vitro, however, conflicting results have been reported regarding the effect of Wnt4 recombinant protein or Wnt4 conditioned medium treatment of muscle cells in AChR clustering in vitro (Strochlic et al., 2012; Zhang et al., 2012). For these experiments, we used a mouse muscle cell line that carries a temperature-sensitive large T oncogene and was generated in our laboratory. Stages of muscle differentiation have been previously described (see M&M, Cartaud et al., 2004; Sigoillot et al., 2010; 2016). We quantified Wnt11 mRNA expression with RT-PCR at three different muscle cell stages. These data revealed that, similarly to Wnt4 (Strochlic et al., 2012), Wnt11 mRNA levels were strongly upregulated at T2 (when AChR clusters are observed) compared to T1 (early formed myotubes without AChR clusters) and then downregulated at T3 (matured contracting myotubes with AChR and acetylcholinesterase clusters). Thus Wnt11 is expressed by muscle cells when AChR clusters begin to form (Figure 1A). T2 muscle cells were treated for 16h with concentrations of Wnt4 or Wnt11 ranging from 2.5 ng/ml to 20 ng/ml. Treatment of muscle cells using 2.5 ng/ml of Wnt4 and/or Wnt11 did not result in a statistically significant effect in AChR clustering (Figure 1B). However, each Wnt induced a significant, dose-dependent increase in AChR clustering beginning at 5 ng/ml with maximal AChR clusters per myotube reached at 10 ng/ml (62% for Wnt4 and 73% for Wnt11; Figure 1B). Importantly, when Wnt4 and Wnt11 were applied together, a significant increase in the number of AChR clusters (~ 45% for 5ng/ml; ~ 80% for 10 ng/ml and ~ 50% for 20 ng/ml) was observed compared to myotubes
treated with Wnt4 or Wnt11 separately. In addition, combined suboptimal doses of each Wnt (2.5 ng/ml) did not induce a larger increase in the number of AChR clusters than when Wnt4 or Wnt11 were applied separately (5 ng/ml) (Figure 1B). These results indicate that both Wnts act cooperatively to induce AChR clustering and suggest that Wnts effects on AChR clustering vary upon the Wnt concentration used. This may help explain some of the reported differences in Wnts on AChR clusters where the concentrations were not carefully controlled.

We further quantified morphological aspects of AChR clusters following Wnt4 and/or Wnt11 treatment overnight at maximal concentration (10 ng/ml; Figure 1C and 1D). Wnt4 and/or Wnt11 treatment similarly enhanced the size of AChR clusters compared to control myotubes (Figure 1C). To assess the specificity of Wnt4 and Wnt11-induced AChR clustering, myotubes were treated overnight with Wnt3 (10 ng/ml), known to have no effect on basal AChR clustering in cultured myotubes (Henriquez et al., 2008). As expected, Wnt3 did not affect the number of AChR clusters compared to control myotubes (Figure 1E and 1F).

We then asked whether both Wnts stimulate AChR clustering in vivo. To directly target the embryonic diaphragm, we used ultrasound-guided injections of Wnt4 and/or Wnt11 in live mice embryos (see M&M and Figure S1) which allowed us to specifically assess in vivo a protein function in a restricted time and spatial frame window (Nieman and Turnbull, 2010; Slevin et al., 2006). A single injection of each Wnt (50 µg/ml) or together (25 µg/ml) was performed directly into the peritoneum of E12.5 embryos to target the diaphragm before NMJs started to form. NMJs were analyzed two days later (E14.5) to visualize the in vivo effects of the injected molecule during the early process of NMJ formation. Whole-mount diaphragms were labeled with α-bungarotoxin (BTX) to detect AChR clusters, together with a mixture of antibodies against synaptophysin (Syn) and neurofilament (NF) to visualize nerve terminals and axonal branches respectively (Figure 2A and 2E). Compared to PBS-injected
controls, injection of Wnt4 or Wnt11 alone did not enhance AChR clustering. However, Wnt4/Wnt11-injected embryos displayed increased number (+131%) and volume (+44%) but not fluorescence intensity of AChR clusters (Figure 2B-D). Moreover, in PBS-injected embryos synapses were concentrated in a narrow band and AChR clusters appeared as a thin line in the middle of each hemidiaphragm, whereas in Wnt4/Wnt11-injected embryos most of AChR clusters were distributed in a 1.3-fold wider area (Figure 2E and 2F). While injection of Wnt4 or Wnt11 alone did not affect nerve terminal arborization and axonal outgrowth (data not shown), injection of both Wnt4 and Wnt11 increased the mean axon length by 44% without affecting the number of primary and secondary branches (Figure 2E and 2G-I). Collectively, these data support a cooperative function of Wnt4 and Wnt11 in AChR clustering and axonal outgrowth.

**Loss of Wnt function impairs NMJ formation.**

Our results prompted us to study the effect of loss of Wnt function during NMJ formation. We had previously demonstrated that $Wnt4^{-/-}$ mice embryos exhibit NMJ formation defects associated with decreased number of prepatterned AChR clusters and increased axon outgrowth within the diaphragm (Strochlic et al., 2012). Thus, we asked whether $Wnt11^{-/-}$ mice embryos display similar NMJ formation defects. Whole-mount diaphragms of E14 and E18.5 $Wnt11^{-/-}$ embryos and WT littermates were labeled with BTX together with a mixture of anti-SYN and anti-NF antibodies (Figure 3A and 3D). In E14 $Wnt11^{-/-}$ embryos, the nerve trunk and branches of the phrenic nerve were normally localized in the central region of the muscle as in E14 WT embryos (Figure 3A), indicating that phrenic axons were able to reach the central and prospective synaptic region of the diaphragm in absence of Wnt11. However, the number of AChR clusters was reduced by 69% compared to WT (Figure 3B). In addition, axons were increased in length by 124% in $Wnt11^{-/-}$ compared to WT (Figure 3C). In E18.5
WT embryos, synapses were concentrated in a narrow band and AChR clusters appeared as a thin line in the middle of each hemidiaphragm (Figure 3D), whereas in Wnt11-/- embryos, AChR clusters were distributed in a 1.9-fold wider area (Figure 3D and 3E). We observed a 30% and a 42% decrease in cluster number and volume, respectively in Wnt11-/- compared to WT (Figure 3F-H). In addition to these postsynaptic defects, the presynaptic component was disturbed in Wnt11-/- embryos (Figure 3D). We found a 63% and a 40% decrease in the number of primary and secondary nerve branches respectively (Figure 3I and 3J). Moreover, instead of ending next to the main phrenic nerve trunk, both primary and secondary branches extended further away from the nerve trunk, with an increase in their length of 112% and 302%, respectively (Figure 3K and 3L). Despite this phenotype, nerve terminals were consistently apposed to AChR clusters in Wnt11-/- embryos indicating that Wnt11 is not involved in synapse recognition.

To investigate the effect of Wnt signaling during NMJ formation and to avoid potential redundancy between distinct Wnt ligands, we injected E12.5 mouse embryos with Sfrp4 (secreted Frizzled related protein 4, 100 µg/ml), a Wnt antagonist which serves as a soluble decoy Wnt receptor and thus inhibits all Wnt function and signaling (Ehrlund et al., 2013; He et al., 2005; Park et al., 2008; Surendran et al., 2005). Analysis of NMJ phenotype in Sfrp4-injected embryos at E14.5 revealed that AChR clusters were distributed in a very thin line in the middle of each hemidiaphragm compared to PBS-injected embryos (Figure 3M). The endplate band width was reduced by 22% in Sfrp4-injected embryos compared to PBS-injected ones (Figure 3N). In addition, the number (-36%), volume (-24%) and intensity (-15%) of AChR clusters were significantly reduced in Sfrp4-injected embryos (Figure 3O-Q). Interestingly, Sfrp4-injected embryos displayed a drastic loss of terminal arborization. Quantitative analysis revealed that the number of secondary branches in close proximity of the nerve trunk was reduced by 52% and the mean axon length was decreased by 36% in
Sfrp4-injected embryos, in contrast to Wnt4 or Wnt11 knock-out mice (Figure 3R-T). Collectively, these data indicate that Wnt proteins contribute to AChR clustering, presynaptic branching and axon outgrowth.

**Wnt4 and Wnt11 signal through the canonical pathway to elicit AChR clustering and NMJ differentiation.**

Wnt4 and Wnt11 are known to activate both canonical and non-canonical Wnt signaling, depending on cell and tissue context (Heinonen et al., 2011; Lyons et al., 2004; Oteiza et al., 2010; Tao et al., 2005; Toyama et al., 2010). In the canonical pathway, Wnt ligands interact with Fzd receptors and Lrp5/6 co-receptors leading to the neosynthesis of dephosphorylated β-catenin or to the stabilization of cytoplasmic β-catenin which is then dephosphorylated and translocated to the nucleus in order to initiate the transcription of Wnt-target genes (Clevers and Nusse, 2012). To evaluate the potential role of Wnt4 and/or Wnt11 on canonical signaling activation in muscle cell cultures, we quantified β-catenin translocation to the nucleus, a classical readout of the canonical Wnt signaling. Both Wnt4 and/or Wnt11 treatment (at maximal concentration for 16h) resulted in increased levels of cytoplasmic β-catenin and a strong increase of the levels of nuclear β-catenin (Figure 4A, 4B and 4D). We also observed a significant decrease of phosphorylated β-catenin protein levels in the cytoplasm when myotubes were treated simultaneously with Wnt4 and Wnt11 (Figure 4A and 4C). Therefore, our data suggest that active β-catenin (non phosphorylated) is stabilized and translocated to the nucleus in response to Wnt treatment. Moreover, both Wnts significantly increased the fluorescence intensity level of nuclear β-catenin (Figure 4E and 4F). Taken together, these results demonstrate that in muscle cells, Wnt4 and Wnt11 signal through the canonical pathway.
We next asked whether canonical signaling induced by Wnt4/11 was required for AChR clustering. We used the secreted Wnt antagonist Dickkopf-1 (Dkk1), a specific inhibitor of canonical signaling, that binds to Lrp co-receptors, including Lrp4, thus competing with Wnt ligands (Choi et al., 2009; Cruciat and Niehrs, 2013; Niehrs, 2006). Dkk1 treatment reduced the number of Wnt4 and/or Wnt11-induced AChR clusters in a dose dependent manner with a maximal effect observed at 20 ng/ml (-26%, -36% and -57% for Wnt4, Wnt11 and Wnt4/Wnt11-induced AChR clusters respectively; Figure 4G and 4H). In addition, Dkk1 (20 ng/ml) impaired nuclear accumulation of β-catenin in myotubes treated with Wnt4 and/or Wnt11, confirming Dkk1 inhibitory effect (Figure S2).

In the canonical pathway, β-catenin can function as a transcriptional co-activator through its association with TCF/LEF transcription factors to induce target gene transcriptional programs (MacDonald et al., 2009; Nusse, 2012). We reasoned that Wnt4 and Wnt11 could regulate AChR clustering in part through the canonical-dependent transcriptional activation of AChR subunit genes. Consistent with this hypothesis, most of the AChR subunit mRNA levels (α, β and γ) were increased in myotubes treated with Wnt4 and Wnt11 compared to control myotubes (Figure 4I). Moreover, Dkk1 (20 ng/ml) treatment abolished Wnt-induced increased levels of these AChR subunit mRNA expression (Figure 4I). Of note, ε AChR subunit mRNA level was not regulated by Wnt or Dkk1 treatment suggesting that this subunit is not a Wnt responsive target gene.

To further assess the role of the Wnt canonical signaling in NMJ formation in vivo, we performed injection of Dkk1 (100 µg/ml) in live embryos (Figure 4J). AChR clusters were spread in a wider muscle area (+16%, Figure 4K) and the number (-29%) and volume (-24%), but not the fluorescence intensity of AChR clusters were significantly reduced in Dkk1-injected embryos compared to E14.5 PBS-injected controls (Figure 4L-N). Notably, Dkk1 injections led to aberrant extension (+69%) of motor axons beyond AChR clusters (Figure
although the number of primary and secondary branches was not affected (Figure 4O and 4P). Collectively, these data indicate that the canonical signaling is required downstream of Wnt4 and Wnt11 to regulate AChR clustering and motor axon outgrowth within the diaphragm. Strikingly, however, Dkk1-injected NMJ phenotype did not recapitulate Sfrp4-injected NMJ defects suggesting that in addition to the canonical signaling, another pathway is required.

The core PCP component Vangl2 accumulates at developing NMJ, interacts with extracellular Wnt4 and Wnt11 and Vangl2 looptail mutation affects NMJ formation

Given the involvement of Wnt11 in the Wnt/PCP pathway (Gao, 2012), we investigated the role of PCP signaling at the NMJ. We focused on Vangl2 (Van Gogh-like 2), one of the most upstream of the core PCP components. Vangl2 is expressed in developing motor axons and is essential for axon guidance in the central nervous system (Avilés and Stoeckli, 2016; Ezan and Montcouquiol, 2013; Nagaoka et al., 2014; Shafer et al., 2011; Tissir and Goffinet, 2013). Moreover, Vangl2 was previously shown to be a postsynaptic protein crucial for synaptogenesis in hippocampal cultures (Nagaoka et al., 2014; Nagaoka et al., 2015). First, we analyzed the Vangl2 pattern of expression during NMJ formation. We found that, Vangl2 mRNA was highly expressed in diaphragm and hindlimb muscles at E14, when NMJs start to form, and decreased as muscle differentiation proceeds (Figure 5A). In addition, we identified Vangl2 in protein extracts of E18.5 brain, spinal cord, diaphragm, hindlimb and myotube cultures (Figure 5B). Moreover, Vangl2 colocalized with both BTX and the SNARE protein SNAP25, a well-known marker of the presynaptic compartment (Söllner et al., 1993; Washbourne et al., 2002) in E18.5 hindlimb sections suggesting that Vangl2 is accumulated at embryonic NMJ (Figure 5C). We then tested the potential interaction between Vangl2 and Wnt4 or Wnt11 in a co-culture co-immunoprecipitation assay
in which the two partners are expressed by different cells allowing the detection of extracellular interactions (Yamamoto et al., 2008). Immunoprecipitation was performed after 24 hours of co-culture between NIH 3T3 fibroblasts transfected with a plasmid coding for GFP-Vangl2 and HEK293T cells expressing Wnt11-myc or Wnt4-HA (Figure 5D). We found that both extracellular Wnt4 or Wnt11 co-immunoprecipitated with Vangl2, suggesting that these three proteins are part a common signaling cascade (Figure 5E).

To assess the structural consequences of Vangl2 deletion on NMJ formation in vivo, we examined the NMJ phenotype of mice embryos bearing the Vangl2 looptail mutation (Vangl2<sup>Lp/Lp</sup>, Figure 6A and 6D) characterized by a point mutation in the Vangl2 gene (S464N) rendering the protein ineffective and unstable (Wang et al., 2005). Early during NMJ formation, in E14/E14.5 Vangl2<sup>Lp/Lp</sup> embryos, the number of AChR clusters was reduced by 39% compared to WT indicating that Vangl2 is required for normal level of AChR clustering at this early stage (Figure 6B). Moreover, axons were increased in length by 191% in Vangl2<sup>Lp/Lp</sup> embryos (Figure 6C). At later developmental stage (E18.5), AChR clusters were distributed over a wider area and the endplate band width was 1.7-fold larger than WT littermates (Figure 6D and 6E). The number (-34%) and volume (-29%), but not the fluorescence intensity of Vangl2<sup>Lp/Lp</sup> AChR clusters were reduced compared to WT (Figure 6F-H). All AChR clusters were innervated in Vangl2<sup>Lp/Lp</sup> (Figure 6D). However, although the number of both primary and secondary axons was not affected (Figure 6I and 6J), Vangl2<sup>Lp/Lp</sup> embryos showed a striking overextension of secondary motor axon branches (+108%), bypassing AChR clusters and growing aberrantly toward the periphery of the muscle (Figure 6K and 6L).

Since the atypical seven-pass cadherin Celsr3, another core component of the PCP signaling pathway, has been recently involved in limb motor axon guidance (Chai et al., 2014; Chai et al., 2015), we wondered whether it could also play a role in NMJ formation. However, E18.5
Celsr3 deficient (Celsr3−−) mice embryos exhibited NMJ phenotype similar to that of WT littermates with no pre- or postsynaptic defects (Figure S3), suggesting that Celsr3 is dispensable for NMJ formation in the diaphragm muscle. As Celsr3 mutant mice show a hindlimb phenotype reminiscent of congenital talipes equinovarus (Chai et al., 2015), one possible explanation is that Celsr3 could function in a subset of motoneurons innervating the hindlimb but not the diaphragm. Alternatively, since three Celsr genes/paralogs exist in mammals, the loss of Celsr3 expression might not be sufficient to alter diaphragm NMJ formation due to redundant Celsr function (Tissir and Goffinet, 2013).

In summary, our data demonstrate that Wnt ligands including Wnt4 and Wnt11 contribute to NMJ formation process via downstream activation of two pathways, the canonical and a Vangl2-dependent core PCP signaling to regulate AChR clustering and key aspects of presynaptic differentiation such as axon branching and outgrowth (Table 1).
**Discussion**

In this study, we provide evidence that pre- and postsynaptic NMJ differentiation requires a balance of distinct Wnt signaling activities mediated in part by Wnt4 and Wnt11. Based on our findings, we propose that the coordinated action of Wnt4 and Wnt11 activates two downstream signaling, the β-catenin-dependent and the Vangl2-dependent PCP to stimulate AChR clusters formation in the postsynaptic muscle membrane and regulate presynaptic motor axon outgrowth (Table 1). In addition, our data show that Wnt11 plays a role in presynaptic branching. Our results further highlight the emerging role of Wnt signaling in mammalian NMJ formation and point to distinct roles of Wnt ligands and associated downstream signaling in regulating various aspect of presynaptic differentiation. This is in line with recent advances in Wnt signaling indicating that the same Wnt can activate different pathways and that several Wnts can mediate one signaling depending on the cell context (Anastas and Moon, 2013).

**Coordinated roles of Wnt4 and Wnt11 in AChR clustering**

Here, we demonstrate that Wnt4 and Wnt11 cooperate to potentiate their individual postsynaptic activity in muscle cells *in vitro* and in E14.5-injected diaphragm *in vivo*. In contrast, loss of Wnt11 or Wnt-induced signaling in mice severely impairs the early phase of AChR clustering with a drastic reduction of AChR clusters. Wnt11 mutant mice were first analyzed at stage E14 and injected embryos were observed at stage E14.5, two days following injection of the molecule (E12.5) to allow full effect of the injected molecule and to visualize the impact of the injected molecule on the early steps of NMJ formation. E14/E14.5 stages correspond to the end of the prepatterning step when nerve terminals have reached their muscle target and started to branch (Tintignac et al., 2015). During this step, early nerve-induced signaling is occurring, many AChR clusters are not yet innervated and both aneural
and neural AChR clusters can be visualized and quantified, thus the AChR defects observed are likely to result from initial nerve-independent postsynaptic differentiation. In line with this, Granato and colleagues showed that both Wnt11r and Wnt4a are required for AChR prepatterning in zebrafish and we previously reported that Wnt4 participates in aneural AChR clustering in mice (Gordon et al., 2012; Strochlic et al., 2012). However, only the combined inactivation of \textit{wnt11r} and \textit{wnt4a} in zebrafish led to a complete loss of aneural AChR clusters and loss of Wnt4 function in mice did not fully abolish muscle prepatterning. Like in zebrafish, the lack of Wnt11 triggers a more pronounced prepatterning defect than Wnt4 deficit. However, the role of Wnt4 and Wnt11 during NMJ formation has been recently challenged by the group of S. Burden who reported finding showing that loss of Wnt11 and/or Wnt4 in mice embryos did not induce postsynaptic differentiation defects (Remédio et al., 2016). Although similarly to Remedio et al., our data suggest that neuromuscular synapses are able to form in absence of Wnt4 and/or Wnt11, strong postsynaptic differentiation defects were quantified revealing that Wnt4 and Wnt11 are key regulators of NMJ formation. In addition, we did not observe the formation of ectopic muscle islands within the central tendon of the diaphragm in Wnt11 mutant mice. Since distinct genetic backgrounds have been used to breed the Wnt4 (129Sv/CBA/C57Bl6; Jeays-Ward et al., 2004) and Wnt11 (C57Bl6/CD1; Cohen et al., 2012) strains, difference in strains might explain the discrepancy observed. Indeed, a recent systematic analysis of the phenotype-genotype relationship shows that very different phenotypes can be observed depending on the genetic background for the same null allele (Sittig et al., 2016). In addition, the role of Wnts was investigated using a muscle conditional mutant of Wntless (Wls) in which the secretion of Wnts from muscle is blocked. However, this does not preclude the compensatory secretion of Wnts by other cells. Moreover, several groups have shown that in addition to Wnt4 and Wnt11, other Wnt proteins are able to stimulate AChR clustering in absence (ie. Wnt9a, 9b, 10b and 16) or presence
(Wnt3) of exogenous neuronal agrin in vitro (Barik et al., 2014; Henriquez et al., 2008), suggesting that NMJ formation is likely to require the coordinated function of multiple pro-
synaptogenic Wnts and that Wnt redundant effects and/or compensatory mechanism
dependent on the genetic background may also in part explain why single or double Wnt
deletion in mice induce distinct NMJ phenotypes.

Our results further indicate that Wnt4/Wnt11-induced AChR clustering in part requires
increase levels of AChR subunit gene expression in a β-catenin dependent manner in muscle
cells and that specific inhibition of the canonical pathway in vivo, before NMJ start to form
lead to an increased AChR endplate band and a decreased number and volume of AChR
clusters. These data demonstrate that Wnt canonical-dependent synaptic gene transcriptional
activation is involved in postsynaptic differentiation. Interestingly, mice depleted of β-catenin
in muscles, displayed similar endplate band width enlargement but divergent AChR cluster
size and no defect of postsynaptic differentiation was detected in muscle β-catenin gain of
function (Li et al., 2008; Wu et al., 2012a). All together, these data highlight the critical role
of Wnt-elicited canonical signaling in patterning the prospective synaptic endplate band and
suggest that a critical level of β-catenin signaling activity is required to regulate AChR cluster
formation. In zebrafish, Wnt canonical signaling is not required for AChR clustering and axon
guidance (Gordon et al., 2012). This could be related to an evolutionary divergent role of Wnt
signaling between zebrafish and mice.

Both Wnt4 and Wnt11 interact with the MuSK receptor via its Frizzled (Fzd) like domain
(Jing et al., 2009; Strochlic et al., 2012; Zhang et al., 2012). We previously demonstrated that
deletion of MuSK CRD (MuSK∆CRD) in mice altered Wnt11-mediated AChR clustering
(Messéant et al., 2015). Moreover, MuSK∆CRD embryos, similarly to Dkk1-injected
embryos, exhibited strong defects of postsynaptic differentiation associated with a decrease of
AChR clusters number, a phenotype that was rescued by force-activation of the canonical
signaling using lithium chloride (Messéant et al., 2015). Strikingly, no NMJ formation and function defects were found in another mouse line deleted from MuSK CRD recently generated by the group of S. Burden (Remédio et al., 2016). One given explanation is that our mice overexpress the mutated MuSK which could in part explain the difference in the NMJ phenotype observed (Messéant et al., 2015). However, although we do not know what accounts for the difference between the two mice lines, MuSKΔCRD heterozygous mice embryos and adult did not display any NMJ phenotype (except a slight increase of the axonal length in E14.5 mice embryos, not observed at stage E18.5) indicating that overexpression of the mutated MuSK is not responsible for the NMJ defects observed (Figure S4). In addition, it is likely that, in contrast to our finding, MuSKΔCRD overexpression would lead to increased AChR clustering and synapse formation similarly to previous results obtained by the group of S. Burden (Kim and Burden, 2008).

Interestingly, our results demonstrate that Wnt4 and Wnt11 also interact with the core PCP protein Vangl2 in vitro and that, like MuSKΔCRD, Vangl2<sup>Lp/Lp</sup> embryos display AChR clustering defects suggesting that these four proteins are part of a PCP signaling complex required for AChR accumulation in the postsynaptic membrane. In line with this, Wnt-initiation of muscle prepatternning in zebrafish requires MuSK endocytosis and components of the PCP pathway (Gordon et al., 2012; Lacazette et al., 2003). Alternatively, since most of the Wnt receptor Fzd are expressed in developing diaphragms (Avilés et al., 2014), Vangl2 may signal via a yet unidentified Fzd receptor to regulate AChR clustering.

**Wnt Signaling in presynaptic branching and motor axon growth**

Our results obtained in vivo using specific inhibitors of Wnt signaling suggest that Wnt ligands activate both canonical and non-canonical pathways differentially involved in motor axon outgrowth and nerve branching/arborization. Our data showed that Dkk1-injected
embryos and mice bearing the Vangl2 looptail mutation exhibited similar phenotype with excessive motor axon growth bypassing AChR clusters suggesting that both canonical and Vangl2-dependent signaling affect axon outgrowth. Interestingly, a similar phenotype was observed in MuSKΔCRD (Messéant et al., 2015) as well as in Wnt4 (Strochlic et al., 2012) and Wnt11 deficient embryos but to a lesser extend (no bypassing of AChR clusters in Wnt11−/− embryos), suggesting that both Wnts mediate canonical and Vangl2-dependent signaling to regulate this presynaptic phenotype.

It has been shown that muscle-specific, but not motoneuronal deletion of β-catenin or Lrp4 expression in mice altered presynaptic differentiation and that muscle β-catenin transcriptional activity is required for presynaptic differentiation, indicating that the presynaptic defects observed in Dkk1-injected mice embryos are likely to result from inhibition of a muscle canonical retrograde signaling pathway (Li et al., 2008; Liu et al., 2012; Wu et al., 2012b). Since our data show that Vangl2 accumulates at developing NMJ likely being expressed in both presynaptic nerve terminals and postsynaptic muscle domains, it is possible that a Vangl2-dependent signaling is activated in motor axons to regulate and stop the growth of axons once they have reached their target. In this line, the role of the PCP pathway in Wnt-induced growth of axons has been largely documented (Onishii et al., 2014). However, the similarity between Dkk1 and Vangl2 phenotypes raise the interesting hypothesis that both Vangl2 and β-catenin signaling are in the same pathway. Indeed, endocytosis regulates receptor tyrosine kinase signaling (Goh and Sorkin, 2013) and it has been shown that some of the core PCP proteins are involved in Wnt-induced MuSK internalization and signaling in zebrafish (Gordon et al., 2012). Thus, in this hypothesis, the PCP pathway would act upstream of β-catenin allowing MuSK endocytosis to trigger the activation of the Wnt canonical signaling. Alternatively, we cannot exclude the possibility that Vangl2 signals in the Schwann cell to regulate NMJ formation.
In contrast to Dkk1-injected embryos and Vangl2 mutant phenotypes and similarly to Wnt11 loss of function, transient inhibition of Wnt signaling pathways by Sfrp4, drastically decreases nerve terminal arborization. This suggests that Wnt11 regulates motor axon arborization via a yet unknown Vangl2-independent and non canonical Wnt pathway. Whether Wnt11<sup>−/−</sup> AChR clustering defects induces abnormal presynaptic phenotype or the reverse are to be clarify <i>in vivo</i>, it remains that Wnt11 controls directly the number of AChR clusters in muscle cells. Whereas Vangl2 mutant, Wnt11 deficient mice, Dkk1- and Sfrp4-injected embryos exhibit the same postsynaptic defects, complex effects affecting specifically arborization or axon outgrowth are observed concerning the presynaptic phenotypes. Deciphering the presynaptic Wnt-induced mechanisms will require identifying the receptors of Wnts and the cell-specific signaling cascades.
Materials and Methods

Reagents and Mice.

The use of animals is in compliance with the European Community guidelines (N°A-75-1970). Mutant mice Wnt11⁻/⁻, Vangl2Lp/Lp and Celsr3⁻/⁻ were described previously (Majumdar et al., 2003; Montcouquiol et al., 2003; Tissir et al., 2005; Wang et al., 2002). The Wnt11⁻/⁻ strain background is a mixture of C57Bl6/CD1 (Cohen et al., 2012). Experimental procedures were performed on mutant males and WT littermates. Information on reagents and antibodies are provided in the supplemental Materials and Methods.

SYBR Green RT-PCR.

Total RNAs from cultured muscle cells, diaphragms or hind limb muscles free of bones and skin were extracted and SYBR Green RT-PCR experiments using Wnt11, Vangl2 and AChR α, β, γ and ε subunit primers (Quiagen) were performed as described previously (Strochlic et al., 2012). At least 3 experiments for each muscle cells stage were performed and 6 embryos for each stage were tested.
**Immunoprecipitation and western blot.**

Subcellular protein fractionation was performed following manufacturer’s instructions (Thermo Scientific). Transfection (4µg of each plasmid), co-culture co-immunoprecipitation and western blot analyses were performed as described previously using indicated antibodies (Giese et al., 2012; Strochlic et al., 2012; Yamamoto et al., 2008). Contrary to “regular” transfection/co-immunoprecipitation assay, the co-culture co-immunoprecipitation assay involves the transfection of two potential interactors in two different cell types, then co-culture and immunoprecipitation assay. By doing so, this technique has proven being a simple and reliable method to detect specific protein-protein interactions occurring only in the extracellular space.

Relative signal intensity of total and phosphorylated β-catenin normalized to β-actin was measured using ImageJ software.

**HEK 293T, NIH 3T3 and Muscle cells culture.**

293T HEK and NIH 3T3 cells (ATCC) were cultured in DMEM supplemented with 10% fetal bovine serum, 2 mM glutamine and 2% penicillin/streptomycin (500U) at 37°C in 5% CO₂. The MLCL polyclonal muscle cell line was generated in our laboratory from H-2Kb-tsA58 newborn mice muscle and was cultured as described previously (Cartaud et al., 2004; Sigoillot et al., 2010; Sigoillot et al., 2016). Three stages of muscle cell differentiation were selected for analysis: T1 when cells are mostly myotubes (day 0), T2 (day 2) when AChR clusters are visualized and T3 (day 5) when both AChR and AChE clusters are observed (Guerra et al., 2005). When indicated, recombinant Wnt3, Wnt4 and/or Wnt11 proteins were added to stage T2 myotubes alone or with Dkk1 for 16h.
Immunohistochemistry.

Staining on whole mount diaphragms, isolated muscle fibers, tissue sections and myotubes was performed as described previously (Messéant et al., 2015; Strochlic et al., 2012).

Images acquisition and processing.

Images acquisition and quantitative analysis of NMJs were performed as previously described (Messéant et al., 2015). Details are provided in the supplementary Materials and Methods. For quantification of AChR cluster in culture myotubes, a size threshold was applied to enable the quantification of AChR clusters with a minimal size of 5 µm^2. This new threshold allows the detection of AChR aggregates and not AChR micro-clusters, in contrast to quantification made in a previous study (Strochlic et al., 2012) and explains the discrepancy in the results.

Ultrasound guided microinjection of embryos.

WT C57/Bl6 pregnant mice (E12.5, Janvier) were anesthetized (3% isoflurane in air and maintained at 1.5%), installed on a heating pad and monitoring of respiration frequency, ECG and temperature was performed (Figure S1A and S1B). Pregnant mouse was IP injected with Metacam (1 mg/Kg/BW, Boehringer Ingelheim). A laparotomy was then performed and the uterine horns were gently exteriorized to allow direct visualization of embryos using an ultrasound biomicroscope (VEVO2100, Visualsonics) equipped with a 60 MHz probe (MS-700, Figure S1C and S1D). To ensure contact between the ultrasound probe and the embryos injected, a warm sterile gel was used (aquasonic). Quartz micropipette for in vitro fertilization (MIC-8-0, Origio; outer diameter, 8-10 µm; inner diameter, 6.2-7.0 µm) was used to directly inject (5µl) into the embryo, targeting the peritoneum. Visual observation allowed the identification of any bleeding or not effective delivery of the injected product (Figure S1E).
At least, about three embryos were injected per uterine horn, selecting those that were in ideal positions, to minimize manipulation of the embryos. The maternal abdomen was then closed using surgery staples, and injected animals were kept isolated in heated cages 24h after surgery. Additional injection of Metacam was performed 24h after surgery. Wnt4, Wnt11, Sfrp4 and Dkk1 were injected in E12.5 live embryos. The injected embryos were sacrificed at E14.5 and NMJ morphological analyses were performed. Considering the relatively short timeframe of the injection (2 days), it is unlikely that any potential observed defects detected in the diaphragms would arise from systemic effects.

**Statistical Analysis.**

All data were expressed as means ± SEM. Statistical analyses and graphs were performed with Prism 6.0 (Graphpad) software. Mann-Whitney U-test was used to compare data between two groups. Data of multiple groups were analyzed by one-way ANOVA. Differences were considered significant when $P<0.05$. Each experiment was conducted a minimum of three times.
Author Contributions

J.M. performed experiments, analyzed the data and wrote the manuscript; J.E., P.D. and K.G. performed experiments; C.M., F.L. and G.R. conducted the ultrasound guided microinjection of embryos; F.T., M.M. and N.S. provided analytical tools and commented on the manuscript; C.L. and L.S. supervised the project, analyzed the data and wrote the manuscript.

Acknowledgments

The authors wish to thank C. Medina, E. Delugré, M. Halliez and M. Boex for technical help and S. Carbonetto for proof reading. We thank M. Kim, E. Cohen, S. Lefebvre and JP. Borg for the Wnt11-myc plasmid, Wnt11−/− mice, SNAP25 and Vangl2 antibodies, respectively. This work was supported by the Association Française contre les Myopathies (AFM), the Fondation pour la Recherche Médicale (FRM), the Centre National de la Recherche Scientifique (CNRS), the Institut National de la Santé et de la Recherche Médicale (INSERM), Bordeaux and Paris Descartes Universities.
References


Figures

Figure 1. Wnt4 and Wnt11 cooperatively enhance AChR clustering in vitro.

(A) Real time RT-PCR quantification of relative Wnt11 mRNA expression during myotubes differentiation at three muscle stages (T1, myotubes formation; T2, visualization of AChR clusters; and T3, late muscle differentiation). (B) Quantification of AChR cluster number in control myotubes or treated with increased concentrations of Wnt4 and/or Wnt11 for 16h. (C) Measurements of the average AChR cluster area. (D and E) Examples of myotubes stained with α-BTX upon control or Wnt4 and/or Wnt11 or Wnt3 (10 ng/ml) treatment for 16h and stained with α-BTX. (F) Measurements of the number of AChR clusters/myotube. At least 80 myotubes were analyzed for each condition. ns, non significant; *, # p<0.05; **, ##p<0.01 and ***, ### p<0.001 (# : compared to untreated myotubes); Mann-Whitney U test or one-way ANOVA. Scale bar: in D and E, 20 µm.
Figure 2. Wnt4 and Wnt11 cooperate to enhance AChR clustering and presynaptic axon outgrowth in vivo.

(A) Confocal images of whole mount left hemidiaphragms from E14.5 PBS or Wnt4 and/or Wnt11-injected embryos stained with α-BTX. Inset, high magnification view of AChR clusters. (B-D) Quantitative analysis of the AChR clusters number (B), volume (C) and fluorescence intensity (D). (E) Confocal images of whole mount left hemidiaphragms from E14.5 PBS or Wnt4/Wnt11-injected embryos stained with neurofilament (NF, red) and synaptophysin (Syn, red) antibodies together with α-BTX (green). White dashed lines, synaptic endplate band. (F-I) Quantification of the endplate band width (F), the number of primary (G) and secondary (H) nerve branches and the mean axon length (I). N=5 embryos per condition. ns, non significant; *p<0.05; **p<0.01 and ***p<0.001; Mann-Whitney U test or one-way ANOVA. Scale bar: in D and E, 40 µm.
Figure 3. Loss of Wnt function impairs NMJ formation.

(A) Confocal images of whole mount left hemidiaphragms from E14 WT and Wnt11−/− embryos (NF/Syn, red; AChR clusters, green). (B and C) Quantitative analysis of the E14 AChR clusters number (B) and mean axon length (C). (D) Confocal images of whole mount left hemidiaphragms from E18.5 WT and Wnt11−/− embryos stained as in A. White dashed lines, synaptic endplate band. (E-L) Quantification of the E18.5 endplate band width (E), the AChR clusters number (F), volume (G), fluorescence intensity (H), the number of primary (I) and secondary (J) nerve branches and the mean primary (K) and secondary (L) axon length. (M) Confocal images of whole mount left hemidiaphragms from E14.5 PBS and Sfrp4-injected embryos (NF/Syn, red; AChR clusters, green). (N-T) Quantification of the endplate band width (N), the AChR clusters number (O), volume (P), fluorescence intensity (Q), the number of primary (R) and secondary (S) nerve branches and the mean axon length (T). *p<0.05; **p<0.001 and ***p<0.001. ns, non significant. N=5 embryos per condition. Mann-Whitney U test. Scale bar in the merged image in A and M, 40 μm; in D, 200 μm.
**Figure 4. Wnt4 and Wnt11 signal through the canonical pathway to elicit AChR clustering and NMJ differentiation.**

(A) Subcellular protein fractionation of T2 myotubes treated or not with Wnt4 (10 ng/ml) and/or Wnt11 (10 ng/ml). Representative western blot analyses of cytoplasmic and nuclear fractions of phosphorylated (P-β-catenin T41/S35) or total β–catenin (β-catenin) antibodies. β-actin was used as a loading control and GAPDH as an indication of cytoplasmic purity. (B-C) Quantification of the total β-catenin (B) and the P-β-catenin (C) levels normalized to β-actin levels in the cytoplasmic fraction upon control or Wnt treatment. (D) Quantification of total β-catenin level normalized to β-actin levels in the nuclear fraction upon control or Wnt treatment. (E) Examples of myotubes stained with total β-catenin (red) together with DAPI (blue, nuclei) upon control or Wnt treatment. (F) Quantification of nuclear β-catenin fluorescence intensity level upon control or Wnt treatment. (G) Examples of myotubes stained with α-BTX treated with vehicle or Dkk1 (20 ng/ml) in the absence or presence of Wnt4 and/or Wnt11 (10 ng/ml) for 16h. (H) Quantification of AChR cluster number in myotubes treated or not with increased concentrations of Dkk1 in the absence or presence of Wnt4 (10 ng/ml) and/or Wnt11 (10 ng/ml). (I) Real time RT-PCR quantification of relative α, β, γ and ε AChR subunit mRNA expression in myotubes treated or not with Wnt4 (10 ng/ml) and/or Wnt11 (10 ng/ml) in the presence or absence of Dkk1 treatment (20 ng/ml). (J) Confocal images of whole mount left hemidiaphragms from E14.5 PBS or Dkk1-injected embryos (NF/Syn, red; AChR clusters, green). White dashed lines, synaptic endplate band. (K-Q) Quantification of the endplate band width (K), the AChR clusters number (L), volume (M), fluorescence intensity (N), the number of primary (O) and secondary (P) nerve branches and the mean axon length (Q). *, #p<0.05; **, ####p<0.01 and ###p<0.001. ns and (ns), non significant. # and (ns): compared to untreated conditions. N = 6 embryos per condition.
Mann-Whitney $U$ test or one-way ANOVA. Scale bar: in E, 10 µm; in G, 20 µm and in J, 40 µm.
Figure 5. Vangl2 accumulates at developing NMJ and interacts with extracellular Wnt4 and Wnt11.

(A) Real time RT-PCR quantification of relative Vangl2 mRNA expression during diaphragm and hindlimb development. (B) Western blot analyses using Vangl2 antibodies in E18.5 brain, spinal cord, diaphragm, hindlimbs and muscle cells extracts. GAPDH was used as a loading control. Black lines indicate that intervening lanes have been spliced out. (C) Examples of E18.5 hindlimbs muscle cross sections stained with SNAP25 (red or green) and/or Vangl2 (red) antibody together or not with α-BTX (AChR, green). (D) Schematic representation of the Wnt4/Wnt11/Vangl2 co-culture co-immuno-precipitation experiment. (E) Co-
immunoprecipitation of Wnt11-myc and Wnt4-HA together with GFP-Vangl2 in NIH 3T3/HEK 293T co-culture. Western blot using GFP, HA and myc antibodies were performed on cell lysates to assess the expression of GFP-Vangl2, Wnt4-HA and Wnt11-myc. **p<0.01. One-way ANOVA.
Figure 6. \textit{Vangl2}^{Lp/Lp} mice embryos display NMJ formation defects.

(A) Confocal images of whole mount left hemidiaphragms from E14/E14.5 WT and \textit{Vangl2}^{Lp/Lp} embryos (NF/Syn, red; AChR clusters, green) (B and C) Quantification of the E14/E14.5 AChR clusters number (B) and mean axon length (C). (D) Confocal images of whole mount left hemidiaphragms from E18.5 WT and \textit{Vangl2}^{Lp/Lp} embryos (NF/Syn, red; AChR clusters, green). White dashed lines, synaptic endplate band. (E-L) Quantification of the E18.5 endplate band width (E), the AChR clusters number (F), volume (G), fluorescence intensity (H), the number of primary (I) and secondary (J) nerve branches and the mean primary (K) and secondary (L) axon length. *p<0.05, **p<0.01 and ***p<0.001, ns, non significant. N = 6 embryos per genotype, Mann-Whitney U test. Scale bar in the merged image in A and D, 40 µm.
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Diagrams summarize the NMJ morphological phenotypes of Wnt4/Wnt11, Sfrp4 and Dkk1-injected, Wnt11−/−, Vangl2 lp lp and Celsr3−/− mice embryos. ↑, increased; ↓, decreased; - , unchanged; I, primary nerve branches; II, secondary nerve branches; Green, AChR clusters; Red, phrenic nerve. Black dashed lines delimit the synaptic endplate band width and include most AChR clusters.
Supplemental Materials and Methods

Reagents and antibodies

The following antibodies and reagents were used: α-bungarotoxin Alexa Fluor® 488 conjugate (Life Technologies, 1/1000), anti-synaptophysin (Life Technologies, 1/5), anti-neurofilament 68 kDa (Chemicon, 1/750), anti-SNAP25 (1/500, Sigma), anti-HA (1/2500; Abcam), anti-β-catenin (Life technologies, 1/1000), anti-phospho β-catenin (1/500, Abcam); anti-β-actin (1/3000); anti-GAPDH (1/5000); anti-GFP (1/500, Roche); anti-myc (1/500; Cell signaling). Dkk1, Sfrp4, Wnt3, Wnt4 and Wnt11 proteins were purchased from R&D system. Polyclonal (1/500 for western blot) and monoclonal (1/200 for immunostaining) anti-Vangl2 have been previously described (Montcouquiol et al., 2006; Puvirajesinghe et al., 2016).

Images acquisition and processing.

All images were collected on a microscope (model BX61; Olympus) equipped with a Fast 1394 Digital CCD FireWire camera or on a confocal laser scanning microscope (Zeiss LSM-710). The same laser power and parameter setting were applied to ensure fair comparison between WT and mutant muscles. Confocal images presented are single-projected image derived from overlaying each set of collected Z-stacks. For quantification of the AChR clusters number, volume and intensity, image stacks were quantified using the ImageJ (version 1.46m) plugin “3D object counter” (Bolte and Cordelières, 2006). The threshold intensity was set by visual inspection of AChR clusters, being the same between WT and mutant images. The endplate band width was defined by the distance between the two farthest AChR clusters from the main nerve trunk. Around 100 measurements regularly spaced and covering the entire diaphragm were taken. For presynaptic quantification, the number and length of primary and secondary branches were performed from at least 6 single-projected
images per genotype using ImageJ software. Primary and secondary branches were defined as axons that extended from the main nerve trunk (primary) or from the primary branches (secondary). For analyses of E14/E14.5 injected-embryos, since secondary branches were not fully differentiated, we quantified the mean axon length defined as the total length of the axons that extended from the nerve trunk. At least 4 diaphragms or 50 isolated muscle fibers of each genotype were analyzed and quantified. To evaluate β-catenin translocation to nuclei in myotubes, image stacks corresponding to nuclei were used for quantification using the ImageJ intensity plot profile.

Supplemental references


Figure S1. Setup for ultrasound-guided injections in live mice embryos.

(A) Photography showing the heating pad on which the anesthetized pregnant mouse was installed and the ultrasound biomicroscope. Additional eating of the animal was ensured with an infrared eating light all along the experiment. (B) Monitoring respiration frequency, ECG and temperature of the mouse was performed. (C) A laparotomy was performed and the uterine horns were gently exteriorized. (D) A warm sterile gel was used to ensure contact between the ultrasound probe and the embryos (E12.5). Quartz micropipette for *in vitro*
fertilization was used to inject into the embryo together with the Visualsonics microinjection positioning system. (E) Example of an ultrasound image showing a microinjection performed directly in the embryo peritoneum. Note that the peritoneum is identified depending on the location of the liver.
Figure S2. Dkk1 impairs β-catenin nuclear accumulation in myotubes treated with Wnt4 and/or Wnt11.

Examples of myotubes stained with total β-catenin (red) together with DAPI (blue, nuclei) treated with Dkk1 (20 ng/ml) in the absence or presence of Wnt4 and/or Wnt11 (10 ng/ml). Scale bar in the merged image, 10 μm.
Figure S3. Celsr3 is dispensable for NMJ formation in diaphragms.

(A) Confocal images of whole mount left hemidiaphragms from E18.5 WT and Celsr3−/− embryos stained with neurofilament (NF, red) and synaptophysin (Syn, red) antibodies together with α-BTX (AChRs, green). (B-I) Quantitative analysis of the endplate band width (B), the AChR clusters number (C), volume (D), fluorescence intensity (E), the number of primary (F) and secondary (G) nerve branches and the mean primary (H) and secondary (I) neurite length. Data are shown as mean ± SEM. ns, non significant. N = 5 embryos per genotype, Mann-Whitney U test. Scale bar in the merged image in A, 50 μm.
Figure S4.
Figure S4. Identical NMJ phenotypes between WT and MuSK⁺/ΔCRD mice during synapse formation and maturation.

(A) Confocal images of whole mount left hemidiaphragms from E14/E14.5 WT and MuSK⁺/ΔCRD embryos stained with neurofilament (NF, red) and synaptophysin (Syn, red) antibodies together with α-BTX (green). (B and C) Quantitative analysis of the E14/E14.5 AChR clusters number (B) and mean neurite length (C). (D) Confocal images of whole mount left hemidiaphragms from E18.5 WT and MuSK⁺/ΔCRD embryos stained as in A. White dashed lines delineate the synaptic endplate band. (E-L) Quantitative analysis of the E18.5 endplate band width (E), the AChR clusters number (F), volume (G), fluorescence intensity (H), the number of primary (I) and secondary (J) nerve branches and the mean primary (K) and secondary (L) neurite length. (M) Confocal images of whole mount isolated muscle fibers from P20 and P60 WT and MuSK⁺/ΔCRD Tibialis Anterior, stained as in A. (N-P) Quantitative analysis of the AChR cluster area (N), the Syn area (O), and overlap area of presynaptic and postsynaptic elements (P) in P20 WT and MuSK⁺/ΔCRD mice. (Q-T) Quantitative analysis of the number of fragments per AChR cluster (Q), the AChR cluster area (R), the Syn area (S), and the overlap ratio of presynaptic and postsynaptic elements (T) in P60 WT and MuSK⁺/ΔCRD mice. *p<0.05; ns, non significant. Mann-Whitney U test. N=4 embryos or animals per condition. Scale bar in the merged image in A and D, 40 μm; in M, 10 μm.