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Afadin Signaling at the Spinal Neuroepithelium Regulates Central Canal Formation and Gait Selection

Graphical Abstract

Highlights
- Afadin elimination from motor neuron progenitors causes hopping phenotype
- Afadin function at the neuroepithelium controls organization of spinal midline
- Miswiring of dI4-5, V0, and V2a premotor interneurons in afadin mutant mice

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In Brief
Skarlatou et al. report that afadin function in the developing spinal neuroepithelium controls organization of the midline. Afadin elimination from the motor neuron progenitor zone results in duplication of the central canal, aberrant wiring of dI4-5, V0, and V2a premotor neurons, and loss of left-right limb alternation.

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Afadin Signaling at the Spinal Neuroepithelium Regulates Central Canal Formation and Gait Selection

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SUMMARY

Afadin, a scaffold protein controlling the activity of the nectin family of cell adhesion molecules, regulates important morphogenetic processes during development. In the central nervous system, afadin has critical roles in neuronal migration, axonal elongation, and synapse formation. Here we examine the role of afadin in development of spinal motor circuits. Afadin elimination in motor neuron progenitors results in striking locomotor behavior: left-right limb alternation is substituted by synchronous activation, characteristic of bound gait. We find that afadin function at the neuroepithelium is required for structural organization of the spinal midline and central canal morphogenesis. Perturbation of afadin results in formation of two central canals, aberrant contralateral wiring of different classes of spinal premotor interneurons, and loss of left-right limb alternation, highlighting important developmental principles controlling the assembly of spinal motor circuits.

INTRODUCTION

The ability to generate coordinated and adaptable movements is essential for survival. In vertebrates, spinal motor circuits orchestrate execution of complex motor programs by integrating descending commands and sensory information to activate biomechanically coherent ensembles of motor neurons in precise sequences (Kiehn, 2006; Goulding, 2009; Arber, 2012). Several classes of interneurons in the spinal cord are involved in wiring of circuits at the basis of motor function, such as coordination of antagonist muscle contraction and left-right alternation of limb movements (Grillner and Jessell, 2009; Gosgnach et al., 2017). Spinal networks controlling the rhythm and pattern of muscle contraction provide a substrate for different locomotor actions ranging from swimming to walking and running. In this context, the relative timing of limb activation is a key element for the control of gait selection in tetrapods (Grillner, 2006; Kiehn, 2016). However, despite the importance of spinal circuits regulating the coordination of limb movement, the principles controlling their assembly and function are not completely understood.

Mouse genetic analysis has revealed specific functions for different spinal interneuron classes in gait control. V0 neurons originating from Dbx1 progenitors have important roles in the coordination of left-right limb movements (Lanuza et al., 2004). Inhibitory V0I neurons have been linked to generation of alternating patterns at low frequencies of locomotion, whereas excitatory V0V neurons control alternation at higher locomotor frequencies (Talpalar et al., 2013). V2a neurons have been shown to influence gait selection depending on the speed of locomotion (Crone et al., 2008, 2009). In addition, roles in coordinating limb activity have been proposed for V3 neurons (Zhang et al., 2008). However, teasing out the precise composition and wiring of spinal motor circuits has been challenging because of the limited ability to selectively access and interrogate the function of discrete neuronal subtypes and circuits in vivo.

Several studies have highlighted the importance of axon guidance molecules such as netrins, slits, and ephrins in directing neuronal connectivity across the neuraxis as a developmental strategy to regulate wiring of spinal circuits (Chedotal, 2019). In particular, ephrin B3 expression at the midline of the hindbrain and spinal cord has been shown to provide a repulsive signal that controls unilateral wiring of EphA4-expressing axons (Kullander et al., 2001). As a consequence, genetic manipulation of ephrin B3-EphA4 signaling results in a dramatic switch from alternation to synchronous activation of limbs (Kullander et al., 2003; Beg et al., 2007; Iwasato et al., 2007). These studies elucidate some of the molecular underpinnings controlling the development of circuits for left-right alternation but also offer an entry point for identification of neurons contributing to these networks. Indeed, selective ablation of EphA4 in spinal excitatory interneurons has been shown to be sufficient to perturb left-right alternation (Borgius et al., 2014). Moreover, elimination of EphA4 expression in spinal interneurons derived from the Lbx1 progenitor domain results in their aberrant bilateral wiring and in a
Figure 1. Loss of Left-Right Limb Alternation in Afadin Mutant Mice

(A) Circular plots showing the phase relationship of fore- and hindlimb movements and the average orientation vector of three individual control mice. The gray area indicates alternation and the orange area synchrony.

(B) Circular plots showing the phase relationship of fore- and hindlimb movements and the average orientation vector of three individual afadin^{fl/fl} mice with the "alternating" phenotype.

(C) Circular plots showing the phase relationship of fore- and hindlimb movements and the average orientation vector of three individual afadin^{fl/fl} mice with the "mixed" alternating and synchronous phenotype.

(legend continued on next page)
context-dependent switch to left-right synchrony (Satoh et al., 2016).

In this study, we set out to investigate the consequences of afadin elimination for the development of spinal motor circuits. Afadin, a scaffold molecule coordinating adhesive function of the nectin and classical cadherin families, has been shown to be involved in the control of neuronal migration and positioning, axonal guidance, and synapse formation (Okabe et al., 2004; Beaudoin et al., 2012; Gil-Sanz et al., 2014; Dewitz et al., 2018). We eliminated afadin from motor neuron progenitors and found that alternation of limb movement is substituted by synchronous activation. To understand the mechanism underlying this phenotype, we studied spinal cord development and assembly of motor circuits in afadin mutant mice. Our data indicate that afadin expression at the neuroepithelium is necessary for lumen formation during development and that its elimination from the motor neuron progenitor zone results in generation of two central canals. As a consequence, the structural integrity of the spinal cord midline is compromised, and the wiring of motor circuits is altered. We identified aberrant contralateral connectivity patterns of dI4-5, V0, V0c, and V2a spinal premotor interneurons, underlining their importance for the control of gait selection.

**RESULTS**

**Loss of Left-Right Limb Alternation in Afadin Mutant Mice**

In previous work, we eliminated afadin from mouse motor neurons by crossing a copy of a floxed allele and a copy of a null allele with the olig2::cre driver line to achieve selective deletion from motor neurons. However, afadin fl/fl; olig2::cre−/− (afadin fl/fl) mice die at perinatal stages because of severe hydrocephalus, precluding analysis of motor behavior (Dewitz et al., 2018). To extend the lifespan, we generated a fully conditional afadin model (afadin fl/fl; olig2::cre+/− mice, hereafter referred to as afadin fl/fl; Figures S1A and S1B). Afadin fl/fl mice are born at expected Mendelian frequency and display a defect in the pattern of activation of left-right limbs during on-ground locomotion.

We performed quantitative gait analysis to precisely characterize the locomotor phenotype of afadin mutant mice (Mendes et al., 2015). Control animals exclusively used alternation of left-right limbs during locomotion, a characteristic feature of a normal walking gait (Figures 1A and 1E; Video S1). In contrast, we observed that one-third of mutant mice mostly used alternating steps but showed a significant increase in the incidence of synchronous ones (Figures 1B and 1E; Video S2), another third presented bouts of alternating and synchronous steps (Figures 1C and 1E; Video S3), and the remaining animals showed constant synchronous activation of left and right limbs (Figures 1D and 1E; Video S4). In this last cohort, step frequency and locomotor speed were significantly increased to values characteristic of bound gait (Figures 1F and 1G; Bellardita and Kiehn, 2015; Lemieux et al., 2016). These data indicate that afadin elimination perturbs the circuits controlling coordination of left-right limb movement, resulting in loss of alternation in favor of synchronous activation (Figure 1H).

**Afadin Mutant Mice Show Abnormal Left-Right Alternation in Isolated Spinal Cord Preparations**

Next we asked whether the phenotype observed in afadin mutant mice depends exclusively on spinal motor circuits and analyzed fictive locomotor activity in isolated lumbar spinal cord preparations from newborn mice (Figure 2A; Kjaerulf and Kiehn, 1996). In control experiments, induction of locomotor-like activity following application of N-methyl-D-aspartate and serotonin produced clear alternating patterns in left (l) and right (r) lumbar (L) roots (Figures 2B and 2C). In agreement with the behavioral data, preparations from afadin fl/fl mice presented a defect in generation of the left-right alternating pattern, ranging from complete synchrony to less penetrant phenotypes characterized by bouts of alternation intermixed with bouts of synchrony (Figures 2D and 2E). In contrast, the pattern of rL2-rL5 roots, which is used as a correlate for flexor-extensor muscle activities, was not affected in mutant spinal cords (Figures 2B and 2D). These experiments suggest that loss of left-right alternation in afadin mutant mice is due to changes in spinal motor circuits and does not require sensory or supraspinal input.

**Afadin Elimination Does Not Affect Motor Neuron Development**

We next considered how afadin elimination influences gait selection. The precision of motor neuron spatial organization in the spinal cord is thought to be an important determinant for assembly of motor circuits (Sürmeli et al., 2011; Bikoff et al., 2016). We previously observed defects in motor neuron positioning in afadin fl/fl embryos; thus, we investigated motor neuron generation, identity, and positional organization in fully conditional afadin mice (Dewitz et al., 2018).

We did not find any disruption in the generation and specification of motor neurons in afadin fl/fl embryos (Figures S1C). Three-dimensional positional analysis was used to assess motor neuron spatial organization, and we observed perturbation in the divisional segregation of the lateral motor column (LMC) in afadin fl/fl embryos (Figures S1D–S1I; Dewitz et al., 2018). Medio-lateral distribution and average settling position analyses showed selective impairment of lateral (LMC) motor neurons migration, as found previously in afadin fl/fl mice, but with a significantly lower phenotypic penetrance (Figures S1J and S1K).

(D) Circular plots showing the phase relationship of fore- and hindlimb movements and the average orientation vector of three individual afadin fl/fl mice with the “synchronous” phenotype.

(E) Average percentage of synchronous steps in control, afadin fl/fl (alt), afadin fl/fl (mix), and afadin fl/fl (sync) mice (mean ± SEM; t test, **p < 0.01, ***p < 0.001; n = 3 for each genotype).

(F) Average speed in control, afadin fl/fl (alt), afadin fl/fl (mix), and afadin fl/fl (sync) mice (mean ± SEM; t test, **p < 0.01, ***p < 0.001; n = 3 for each genotype).

(G) Average step frequency in control, afadin fl/fl (alt), afadin fl/fl (mix), and afadin fl/fl (sync) mice (mean ± SEM; t test, **p < 0.01, ***p < 0.001; n = 3 for each genotype).

(H) Phase distribution histogram for all the steps analyzed in control mice (top panel, n = 3) and afadin fl/fl mice (bottom panel, n = 9).
Next we studied motor neuron organization in adult mice. Because transcription factors defining motor neuron subtype identities are no longer expressed at postnatal stages, we assessed the position of medial and lateral LMC neurons using retrograde labeling of cell bodies by muscle injection of the cholera toxin B subunit (CTB). We chose the gastrocnemius (GS) and tibialis anterior (TA) muscles to label a medial and a lateral motor pool. We found CTB-labeled TA and GS motor neurons at the expected rostral lumbar levels of the spinal cord (Figures S1L and S1M; McHanwell and Biscoe 1981; Sürmeli et al., 2011). Transverse and coronal contour analyses showed that the two pools were clustered and clearly segregated from each other (Figures S1N–S1Q). Accordingly, no significant difference was observed in average settling positions and correlation analysis indicated conservation of motor neuron spatial organization in control and afadin fl/fl mutant mice (Figures S1R–S1T).

Altogether, these data indicate that elimination of afadin from motor neurons causes a developmental delay in their positioning.
that is resolved during late embryonic development and no longer apparent at postnatal stages.

**Afadin Mutant Mice Present a Double Central Canal Phenotype**

The absence of a motor neuron positioning defect in adult *afadin*<sup>fl/fl</sup> mice suggests that the locomotor phenotype does not depend on changes in motor pool organization, raising the question of how afadin elimination affects gait selection. Afadin is ubiquitously expressed in the spinal cord during embryonic development, with higher levels found at the neuroepithelium (Figures 3A–3F and S2A–S2D). Conditional elimination of afadin using the *olig2::cre* allele results in its loss in motor neuron progenitors, suggesting that lack of afadin function at the neuroepithelium might be responsible for the locomotor phenotype (Figures 3I–3L, S2G, and S2H). At E14.5, we observed a clear disruption in the morphology of the midline, resulting in formation of two independent tubular structures above and below the pMN (Figures 3M, 3N, S2I, S2J, and S2P). As a result, a double central canal is evident by P1 and maintained into adult stages (Figures 3O, 3P, S2K, S2L, S2Q, and S2R; data not shown). These data suggest that afadin function at the neuroepithelium is required for central canal formation.

**Afadin Regulates Development of the Spinal Cord Midline**

To examine whether afadin has a general role in the organization of midline structures during development, we generated a conditional mutant using the *Wnt1::cre* line, which recombines in dorsal spinal cord progenitors (*af fl/fl Wnt1*; Rowitch et al., 1999). *Af fl/fl Wnt1* mice die at late embryonic stages as a result of exencephaly. At E14.5, we observed a reduction in the dorso-ventral extent of afadin and phalloidin expression at the neuroepithelium of mutant mice (Figures 4E and S3A–S3D). The defect was significantly more pronounced at E16.5 (Figures 4A–4E). In addition, we found that the extent of the dorsal gray matter at the midline was significantly increased whereas the ventral one was reduced, resulting in a ventral shift in positioning of the developing central canal in *af fl/fl Wnt1* mice (Figures 4A, 4B, 4F–4K, S3E, and S3F). Thus, elimination of afadin from dorsal progenitors also affects anatomical organization of the midline.

Next, to directly test whether afadin function in motor neuron progenitors is required for the double central canal and locomotor phenotypes, we eliminated afadin using the *ChAT::cre* allele, which selectively recombines in postmitotic motor neurons, sparing the progenitor zone (*af fl/fl ChAT*; Rossi et al., 2011). *Af fl/fl ChAT* mice are born at expected Mendelian frequency, do not show any defect in central canal development, and present a normal alternating locomotor pattern (Figures 4L–4P). These data indicate that afadin plays an important role in development and remains expressed around the central canal until adult stages. In mutant embryos, afadin expression is eliminated from the motor neuron progenitor zone (pMN) as early as E10.5 while remaining unaffected in the rest of the neuroepithelium (Figures 3I–3L, S2G, and S2H).

**Afadin Mutant Mice Present a Double Central Canal Phenotype**

(A–H) Afadin expression in E10.5 (A and B), E13.5 (C and D), E15.5 (E and F), and P1 (G and H) control spinal cords. The arrow in (G) points to the central canal. (I–P) Afadin expression in E10.5 (I and J), E13.5 (K and L), E15.5 (M and N), and P1 (O and P) afadin<sup>fl/fl</sup> spinal cords. Arrows in (I) and (K) point to the pMN. Arrows in (M) and (O) point to the central canals.
maintaining neuroepithelial organization along the spinal midline and that its elimination from the pMN results in double central canal formation and perturbation of circuits controlling left-right alternation.

**Midline Expression of Netrin 1 and Ephrin B3 Is Perturbed in Afadin Mutants**

The locomotor and anatomical phenotypes observed in afadin null mice raise the question of whether the signals controlling axonal guidance across the midline are perturbed. Netrin 1 and ephrin B3 are prominent examples of such molecules (Serafini et al., 1996; Kullander et al., 2001). Disruption of netrin 1 or ephrin B3 signaling results in loss of left-right limb alternation (Kullander et al., 2003; Rabe et al., 2009). Thus, we examined the expression of these molecules in afadin null mutant mice.

In control mice, netrin 1 is localized in the ventral part of the midline ranging from the central canal to the white matter.

**Figure 4. Afadin Function at the Neuroepithelium Regulates Development of the Spinal Cord Midline**

(A and B) Transverse sections of an E16.5 control (A) and afadin null Wnt1 (B) spinal cords. Arrows point to the central canal. Scale bar, 100 μm.

(C and D) Afadin and phalloidin expression in the central canal area of E16.5 control (C) and afadin null Wnt1 (D) spinal cords. Bars represent the extent of afadin/phalloidin expression.

(E) Quantification of afadin expression at the midline in E14.5 and E16.5 control and afadin null Wnt1 spinal cords (mean ± SEM; t test, *p < 0.05; n = 3).

(F and G) High magnification of the dorsal midline area in E16.5 control (F) and afadin null Wnt1 (G) spinal cords. Dashed lines delineate the neuroepithelium and the border between the dorsal gray and white matter.

(H) Quantification of the extent of dorsal gray matter in E14.5 and E16.5 control and afadin null Wnt1 spinal cords (mean ± SEM; t test, *p < 0.05; n = 3).

(I and J) High magnification of the ventral midline area in E16.5 control (I) and afadin null Wnt1 (J) spinal cords. Dashed lines delineate the neuroepithelium and the border between the ventral gray and white matter.

(K) Circular plots showing the phase relationship of fore- and hindlimb movements and the average orientation vector in af null ChAT mice (n = 3).

(L) Average speed in control and af null ChAT mice (mean ± SEM; t test, p > 0.05; n = 3).

(M) Average step frequency in control and af null ChAT mice (mean ± SEM; t test, p > 0.05; n = 3).

(N) Average percentage of synchronous steps in af null ChAT mice (mean ± SEM; n = 3).

(P) Afadin expression in E10.5, E13.5, E15.5, and p1 af null ChAT spinal cords. The arrow points to the central canal.
The presence of a double central canal in afadin mutant mice resulted in a reduction in the extent of netrin 1 expression, raising the possibility that development of commissural axons could also be perturbed (Figure 5B). To address this question, we analyzed TAG-1 and ROBO3 expression and localization in the spinal cord of afadin mutant mice, and we did not observe any difference in the trajectory of commissural axons approaching the floor plate or in the thickness of the ventral commissure (Figures 5C–5F and S4A–S4D).

Next, we examined ephrin B3 expression. In control mice, ephrin B3 was localized along the whole dorsoventral extent of the spinal cord gray matter above and below the central canal (Figures 5G). In contrast, in afadin mutant mice, ephrin B3 expression was absent between the two central canals (Figures 5H). Moreover, this phenotype is tightly linked to the central canal defect because localization of ephrin B3 at the midline of af fl/fl ChAT mice was not affected (Figure S4E).

Aberrant Wiring of Premotor Circuits in Afadin Mutant Mice

Ephrin B3 serves as a molecular barrier that prevents EphA4-expressing neurons from sending axons to the contralateral side of the spinal cord (Kullander et al., 2001). The disruption in expression of ephrin B3 raises the possibility that the defect in left-right limb coordination could result from aberrant midline crossing of ipsilaterally projecting neurons expressing EphA4 (Paixao et al., 2013; Borgius et al., 2014). Indeed, we observed an increase in contralaterally projecting axons in afadin fl/fl mice as early as E15.5 (Figures S4F and S4G). Dorsal premotor interneurons originating from the Lbx1 progenitor domain have been shown to aberrantly connect to contralateral motor neurons upon disruption of ephrin B3-EphA4 signaling (Satoh et al., 2016). Thus, we analyzed premotor connectivity using rabies virus (RV) monosynaptic tracing (Wickersham et al., 2007). Expression of RV glycoprotein (G) in motor neurons was obtained using the
The overall distribution of premotor interneurons, we digitally reconstructed their three-dimensional positions and compared their distributions. In control mice, most premotor interneurons were located ipsilaterally in Rexed laminae VI, VII, and X, whereas on the contralateral side, they were mainly confined to lamina VIII, as described previously (Figure 5A). Quantitatively analyze the spatial organization of premotor interneurons in 

We next analyzed the connectivity of Lbx1-expressing premotor interneurons and found an ectopic cluster in the dorsal contralateral side of 

We next checked whether the position of V0c neurons was affected by the presence of a double central canal in 

Multiple Cardinal Interneuron Subtypes Are Miswired in Afadin Mutant Mice

Comparison of total and Lbx1+ premotor interneuron maps in 

First, we focused on interneurons arising from the V0 progenitor domain. In the absence of a defining marker still expressed at postnatal stages (Lu et al., 2015; Ziskind-Conhaim and Hochman, 2017).

We next checked whether the position of V0c neurons was affected by the presence of a double central canal in afadin 

In control mice, we observed Chx10+ premotor interneurons exclusively in the ipsilateral side of the spinal cord (Figures 7Q and 7S). In contrast, contralateral Chx10+ premotor neurons were found in afadin 

DISCUSSION

The precise rhythm and pattern of muscle activation underlie execution of coordinated movements and rely on correct assembly and function of spinal circuits. We found that conditional elimination of afadin in motor neuron progenitors results in loss
Figure 7. Multiple Interneuron Subtypes Are Miswired in Afadin Mutant Mice
(A and B) Contralateral transverse sections of a control (A) and an afadin \textsuperscript{fl/fl} (B) spinal cords showing rabies-labeled interneurons and Lhx1 expression. Insets show magnification of a representative neuron marked by the arrow.

(legend continued on next page)
of left-right limb alternation in favor of synchronous movement. Our data show that afadin function at the neuroepithelium is important for lumen formation and that, in its absence, development of midline structures is perturbed. In particular, afadin elimination from the pMN results in a double central canal phenotype. As a consequence, the anatomical organization of the spinal midline is disrupted. Analysis of premotor circuit assembly shows the emergence of ectopic clusters of contralateral premotor interneurons in afadin mutant mice. In particular, our data indicate that miswiring of multiple subtypes, including dl4-5, V0, V0c, and V2a identities, is necessary to cause loss of left-right limb alternation.

**Afadin Is a Key Regulator of Lumen Formation**

Generation of tubular structures is a morphogenetic process essential for functional organization of many organs and relies on establishment of epithelial polarity and actin cytoskeleton remodeling (Datta et al., 2011). Afadin is known to have important roles in epithelial cells in the formation of adherens and tight junctions as well as in the organization of apicobasal polarity (Mandai et al., 2013). In the developing kidney and pancreas, afadin has been shown to be a central element in the generation and elongation of epithelial tubules. Lumen formation in the kidney requires alignment of apical membrane domains on adjacent epithelial cells that coalesce to generate a lumen, and, upon afadin elimination, nephrons develop a discontinuous lumen (Yang et al., 2013). Similarly, formation of uninterrupted pancreatic lumens occurs via apical membrane biogenesis in a highly coordinated process between epithelial cells that depends on afadin function (Azizoglu et al., 2017). Our findings in the spinal cord are in line with these observations. Elimination of afadin from the pMN creates a gap between adjoining neuroepithelial areas at the midline, precluding their coalescence and causing formation of two independent luminal structures. Moreover, elimination of afadin from dorsal progenitors expressing Wnt1 also perturbs organization of the midline and results in displacement of the central canal to a more ventral position, highlighting the important role of afadin along the whole dorso-ventral extent of the spinal neuroepithelium.

Lumen morphogenesis in the pancreas has been shown to rely on the cooperation of afadin and RhoA activities in the control of cytoskeletal remodeling (Azizoglu et al., 2017). In the spinal cord, previous work showed that elimination of RhoA from the neuroepithelium leads to defects in midline development, indicating that the same signaling pathway controls tubulogenesis in different organs and raising the question of whether specific cell surface receptors are required for this function (Katayama et al., 2012; Mulherkar et al., 2013). Afadin is known to support function of the classical cadherin and nectin families of adhesion molecules (Takai et al., 2008; Mandai et al., 2013). We showed that elimination of afadin in motor neuron progenitors does not affect classical cadherin localization and function (Dewitz et al., 2018). The nectin family consists of four members: nectin-1, nectin-2, and nectin-3 are expressed at the spinal neuroepithelium during development, whereas nectin-4 is not detectable (Okabe et al., 2004; Dewitz et al., 2018). We observed perturbation in nectin-1, nectin-2, and nectin-3 expression at the pMN upon elimination of afadin in motor neuron progenitors (data not shown). However, single nectin mutant mice do not present anatomical or locomotor phenotypes similar to the ones found after elimination of afadin (Takai et al., 2008; Mandai et al., 2015). These observations indicate that nectins may have a redundant function, upstream of afadin, in the control of neuro-epithelial integrity in the spinal cord. Finally, afadin has also been implicated in control of the ephrin/Eph and Slit/Robo pathways, raising the possibility that multiple effectors could contribute to the phenotype (Hock et al., 1998; Slovaková et al., 2012).

**The Double Central Canal and Wiring of Spinal Motor Circuits**

How does generation of a double central canal affect wiring of spinal motor circuits? Our data show that perturbation of central canal formation results in altered organization of the spinal cord midline. We found that ephrin B3, which is normally expressed along the entire dorso-ventral extent of the gray matter, is lost between the two canals, suggesting that the tissue in the central area of the spinal cord is not properly organized (Imondi et al., 2000; Kullander et al., 2001). Similarly, the extent of netrin 1 expression along the ventral half of the midline is also reduced (Serafini et al., 1996). Despite these
remarkable anatomical defects, we did not observe any change in the stereotypic positioning of spinal interneurons normally residing in the intermediate zone of the spinal cord, indicating that the generation and migration of interneurons are not affected by formation of a double central canal. Thus, our data suggest that impairment in lumen morphogenesis compromises the organization of midline signals ensuring laterality in circuit wiring.

**Connectivity Defects in Multiple Premotor Neuron Subtypes in Afadin Mutant Mice**

Perturbation of the localization of midline cues controlling guidance of developing axons is predicted to affect laterality in wiring of motor circuits (Kullander et al., 2001; Rabe et al., 2009). Despite the reduction in the extent of netrin 1 expression in the ventral spinal cord in afadin mutant mice, we did not observe any obvious defect in commissural axon organization. Elimination of ephrin B3 expression between the two central canals in afadin mutant mice is predicted to open a way across the midline for EphA4-expressing axons during assembly of spinal circuits. It has been shown that perturbation of EphA4 signaling in interneurons derived from the Lbx1 progenitor domain results in aberrant contralateral projections (Paixão et al., 2013). Lbx1-derived interneurons can be divided into three main subpopulations: d14 and d15 neurons residing in the dorsal spinal cord and ventral d16 neurons (Gross et al., 2002; Müller et al., 2002). In afadin mutant mice, we found Lbx1+ contralateral premotor neurons in the dorsal spinal cord, indicating a role of d14-5 neurons in circuits controlling gait selection. The same population of interneurons has been identified as a source of ectopic contralateral projections to motor neurons in EphA4 knockout mice, and conditional elimination of EphA4 in Lbx1-derived interneurons is sufficient to result in connectivity defects (Satoh et al., 2016). These mice present an alternating gait during on-ground locomotion but conditionally switch to a synchronous gait when performing locomotor tasks demanding less weight load, such as swimming or air stepping. These observations suggest that changes in the connectivity of multiple interneuron subtypes are required to constitutively synchronize paired limb movement.

Our experiments with afadin mutant mice provide important clues for identification of additional defects in premotor connectivity required for a complete switch to synchrony. We found an ectopic premotor cluster of Lhx1+/FoxD3− neurons in the dorsal contralateral spinal cord. These cells reside in a more lateral position compared to the Lbx1+ cluster, indicating that they constitute a different subset of Lbx1-derived neurons or may originate from V0 progenitors (Lu et al., 2015). Interestingly, we did not observe any difference in premotor connectivity of neurons labeled by FoxD3, which comprise mostly V1 inhibitory interneurons that have no described role in left-right alternation (Arber, 2012). A fraction of V0 neurons has been shown to express EphA4 during embryonic development; however, no defects in connectivity across the midline for V0 neurons in mice with perturbed ephrin signaling have been reported yet (Lundfald et al., 2007). We observed an increase in contralateral connectivity of V0c neurons, a small cholinergic subset of V0 neurons that has been shown to bilaterally connect to motor neurons and participate in modulation of motor output (Zagoraiou et al., 2009; Stepień et al., 2010). Thus, our data suggest that incorrect wiring of V0c could also contribute to the deregulation of circuits controlling gait choice. Finally, we found ectopic contralateral premotor neurons characterized by expression of Chx10. V2a neurons are predicted to be a main culprit for the loss of left-right alternation in the absence of ephrin signaling because of their exclusive ipsilateral connectivity, glutamatergic character, and expression of EphA4 (Al-Mosawie et al., 2007; Lundfald et al., 2007; Borgius et al., 2014). Moreover, genetic ablation of V2a neurons results in loss of left-right alternation in a speed-dependent manner (Crone et al., 2009). However, contralaterally projecting V2a neurons have not been observed previously in EphA4-null mice (Lundfald et al., 2007). In contrast, our data also show that laterality in V2a neuron connectivity is compromised in the absence of ephrin signaling and suggest that this defect contributes to the constitutive emergence of a bound gait.

**STAR METHODS**

Detailed methods are provided in the online version of this paper and include the following:

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Supplemental Information can be found online at https://doi.org/10.1016/j.celrep.2020.107741.

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AUTHOR CONTRIBUTIONS

DECLARATION OF INTERESTS
The authors declare no competing interests.

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REFERENCES


## STAR METHODS

### KEY RESOURCES TABLE

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## RESOURCE AVAILABILITY

### Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Niccolò Zampieri (niccolo.zampieri@mdc-berlin.de).

### Materials Availability

All unique reagents generated in this study are available from the Lead Contact without restriction.
Data and Code Availability
Original datasets supporting the current study are available from the Lead Contact upon request. This study did not generate any new code.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animal Experimentation Ethical Approval
All experiments were performed in compliance with the German Animal Welfare Act and approved by the Regional Office for Health and Social Affairs Berlin (LAGeSo) under license numbers G0121/15 and G0122/15.

Animal models
Mice were bred under standard husbandry and housing conditions. The mouse lines were used in this study maintained on a C57B6/J background: Olig2::Cre (Dessaud et al., 2007), ChAT::Cre (Rossi et al., 2011), Wnt1::Cre (Rowitch et al., 1999), afadin fl/fl (Beaudoin et al., 2012) and RhGT (Takatoh et al., 2013). afadin fl/fl and RhGT were kept homozygous whereas all Cre-lines were kept heterozygous. Animals of both sexes were used interchangeably and we did not anticipate nor observe any influence to the outcome. Following ages were used in this study: Afadin fl/fl; Olig2Cre +/- (E10.5-P63). These mice sporadically develop hydrocephalus. Mice with hydrocephalus were sacrificed as soon as the disease was diagnosed); afadin fl/fl; Wnt1Cre +/- (E13.5-E16.5. These mice display exencephaly); afadin fl/fl; ChATCre +/- (E10.5-P21); afadin fl/fl; Olig2Cre +/-; RhGT fl/fl (P4-P10. These mice sporadically develop hydrocephalus. Mice with hydrocephalus were euthanised as soon as the disease was diagnosed).

METHOD DETAILS

Gait analysis
Quantitative gait analysis was performed as previously described (Mendes et al., 2015). Briefly, a customized acrylic glass walkway was constructed and surrounded by LED lights to generate the frustrated total internal reflection effect that is at the basis of the optical touch sensor that tracks the footprints. A lightbox was positioned above the walkway to visualize the body outline of the animals and a mirror was placed at a 45° angle below the walkway to reflect the signal to a high-speed camera. Before data acquisition, animals were habituated to the walkway setup and trained to walk straight from end to end. Three control animals and a total of nine afadin fl/fl mutant mice were analyzed. For each subject, three representative videos with uninterrupted runs at similar velocities were evaluated using the customized open-source MouseWalker software and the quantifiable parameters averaged.

Isolated spinal cord preparations
Postnatal days 0-3 mice were used in all experiments. The pups were anesthetized with isoflurane, decerebrated and the spinal cord was dissected and isolated in ice-cold Ringer’s solution that contained (in mM): 111 NaCl, 3 KCl, 25 NaHCO3, 1.25 MgSO4, 1.1 KH2PO4, 2.5 CaCl2 and 11 D-Glucose, and oxygenated in 95% O2, 5%CO2 to obtain a pH of 7.4. Isolated spinal cords (from T8 to S1) were transferred into a recording chamber that was continuously perfused with the above Ringer’s solution, and pinned to a Sylgard 184 resin. All recordings were done at room temperature (22-25°C). Drug-evoked locomotor-like activities were induced by bath-applying 7-10 μM of N-methyl-D-aspartate (NMDA, Tocris) and 8-10 μM of serotonin (5-HT, Sigma-Aldrich). Neuronal activities were recorded using extracellular suction glass pipettes (120F-10, Harvard Apparatus) attached to the 2nd and 5th lumbar ventral root (rL2, lL2, rL5). Signals were collected and band-passed filtered at 100 Hz to 1 kHz with an AC amplifier (Model 1700, A-M Systems) and live-integrated (Neurolog, Digitimer) with a time constant of 200 ms. Signals were sampled using Clampex 11 (Molecular Devices) at 5 kHz.

Analysis of fictive locomotion data
For each preparation, flexor-extensor coordination was analyzed offline for 40 consecutive locomotor bursts, taken after the locomotor activity had stabilized, i.e., 15-20 min after the initial burst of activity, as previously described (Talpalar et al., 2013). The left 2nd lumbar root was taken as a reference and bursts timings were detected manually using Clampfit (Molecular Devices). The cycle periods of this reference trace were normalized to a scale from 0 to 1, with the onset of a locomotor burst corresponding to a phase value of 0 and the onset of the next burst corresponding to a phase value of 1. The onset of the burst on the contralateral root (rL2) was normalized to this reference cycle and expressed as a fraction from 0 to 1. This value was used to illustrate the L/R root phase in figures, produce distribution histograms, or plotted on circular plots (Kjaerulf and Kiehn, 1996; Talpalar et al., 2011). For each preparation, the preferred mean phase of the activity was calculated. The direction of the corresponding vector gives the preferred phase of the activity and the length of the vector the precision of the phase [upper half circle: synchrony, lower half circle: alternation; (Kjaerulf and Kiehn, 1996; Talpalar et al., 2011)]. P values smaller than 0.05 determined by Rayleigh’s test and represented outside of the inner circle, were considered significant.
Rabies virus production
Production of rabies virus was performed as previously described (Osakada and Callaway, 2013). Briefly, rabies virus production was started by infecting one T175 flask of BHK B7GG cells with stocks of rabies virusΔG-mCherry (RVΔG-mCherry) supernatant with a titer of at least 10^6 virus particles/ml. Cells were kept at 35 °C and 3% CO₂ and medium was collected after four and seven days, centrifuged at 1000 rpm for 5 minutes and filtered. Amplification was started by infecting ten T175 flasks of BHK B7GG cells with the filtered starter culture. Medium was collected after seven, ten and seven days, centrifuged at 1000 rpm for 5 minutes and filtered. To concentrate the virus, the medium was centrifuged at 24000 rpm for 2 hours. Pellets were resuspended in 400 µL Hanks’ balanced salt solution (HBSS), tubes were covered with paraffilm and incubated ON on a shaker at 4°C. Resuspended pellets were pooled and concentrated at 4°C using an Amicon Ultra 4 mL Filter (Merck Millipore) until a volume of approximately 100 µL was reached. Virus suspension was aliquoted and stored at −80°C until further use. Titer was determined by infecting HEK293T with serial dilutions of virus suspension.

Intramuscular injections
For motor pools analysis at adult stages, p60 animals were injected subcutaneously with 5 mg of carprofen per kg of weight as pre-emptive analgesia 30 minutes prior to surgery. Animals were then initially anesthetized with 4% isoflurane and checked for the toe pinch reflex before any procedure was done. Once sedated, they were placed on top of a circulating warm water blanket maintained at 37°C while keeping them under the effect of 2% isoflurane. Eye lubricant was applied to prevent their eyes from drying out. Head and body were gently fixed with tape to minimize movement during the surgery. To avoid any infection during the procedure, the hindlimbs were treated with iodine solution. A small incision was made in the skin to reveal the gastrocnemius (GS) and tibialis anterior (TA) muscles and a glass capillary was used to introduce 1 µL of cholera toxin subunit B (CTB) conjugated to either Alexa Fluor 488 or 555 (Thermo Fisher). Following injection, the skin was sutured with a nylon surgical suture. Animals were sacrificed three days post-surgery.

For monosynaptic tracing analysis P4 animals were anesthetized as described above and injected with 1.5 µL of RVΔG-mCherry in the extensor carpi radialis (ECR) muscle. Animals were sacrificed six days post-surgery.

Perfusion
Animals were anesthetized by intraperitoneal injection of 0.1 mL ketamine/xylazine mix per 10 g of weight (final concentrations: 120 mg/kg and 10 mg/kg, respectively) and checked for toe-pincher reflex before any procedure was done. Animals were then transcardially perfused with ice-cold PBS until the liver was cleared of blood, followed by freshly made ice-cold 4% PFA until no tremors could be observed anymore. Amount of PBS and PFA needed for a complete fixation depended on the age and weight of the animal (approximately 6 mL of each solution were used for neonates and 12-15 mL for adult animals).

Dissection and tissue processing
Spinal cords were dissected by decapitating the animals, opening up the belly and rib cage and removing the visceral organs. For all animals older than E13.5, ventral laminectomy was performed to allow better penetration of the fixative solution. Spinal cords were fixed for 90 minutes in 4% PFA on ice. This was followed by three washes with ice-cold PBS for 5 minutes each and overnight incubation in 30% sucrose in 0.1M PB at 4°C for cryoprotection. Samples were embedded in Optimal Cutting Temperature (O.C.T., TissueTek) compound, frozen on dry ice and stored at −80°C.

Contralateral interneurons tracing
E15.5 embryos were dissected as described above. 0.5 µL of 1% tetramethylrhodamine-dextran solution was injected unilaterally with a glass capillary in the spinal cord between C5 and C8. Injected embryos were incubated in oxygenated 1x ACSF (127 mM NaCl; 3 mM KCl; 1.25 mM NaH2PO4 . 2 H2O; 26 mM NaHCO3; 10 mM glucose; 2 mM CaCl2; 1 mM MgCl2) over night at 4°C. Cell nuclei were stained with 1 µg/mL DAPI. For monosynaptic tracing analysis P4 animals were anesthetized as described above and injected with 1.5 µL of RVΔG-mCherry in the extensor carpi radialis (ECR) muscle. Animals were sacrificed six days post-surgery.

Immunohistochemistry
16 µm or 40 µm spinal cord cryosections were acquired with a Leica cryostat and collected on superfrost microscope slide. Slides were kept at room temperature for approximately 30 minutes to ensure good adhesion of the tissue on the glass. Sections were then first rehydrated with PBS for 10 minutes before adding freshly prepared dilutions of primary antibodies in 0.1% Triton X-100 in PBS (PBX, 16 µm) or 0.3% PBX (40 µm) for over night incubation at 4°C. Primary antibody dilutions were used as follows: guinea pig anti-MBP 1/20000, rabbit anti-ChAT 1/16000, sheep anti-Chx10 1/500, guinea pig anti-FoxD3 1/20000, rabbit anti-Hb9 1/8000, guinea pig anti-Lmx1 1/30000, rabbit anti-Lbx1 1/1000, rabbit anti-Lmx1 1/1000, rabbit anti-Robo3 1/200, goat anti-ROBO1 1/200, goat anti-ROBO3 1/200. After three washes with 0.1% PBX for 5 min each, secondary antibodies diluted in 0.1% or 0.3% PBX were added for 1 hour at room temperature. Alexa Fluor 488- and Cy3-conjugated secondary antibodies were used at 1/1000, Cy5-conjugated secondary antibodies at 1/500. Sections were then washed twice with 0.1% PBX for 5 minutes, followed by a final wash with PBS for 10 minutes. Slides were mounted with Vectashield (Vector). Nissl and phalloidin stainings were performed by adding NeuroTrace (Thermo Fisher) at 1/250 or Phalloidin-FITC (Sigma Aldrich) at 1/2000 along with the secondary antibodies.
In situ hybridization

*In situ* hybridization histochemistry was performed on 12 μm cryosections using digoxigenin (DIG)-labeled cRNA probes. DNA templates for mouse ephrin B3 and netrin 1 were cloned from cDNAs into pBluescript II SK⁺ vector. Antisense DIG-cRNA probes were generated from DNA templates by RT-PCR using a DIG RNA Labeling Kit (Roche).

In situ hybridization was performed as previously described (Demireva et al., 2011). Briefly, slides were treated as follows: air-dried for 30 minutes, fixed with freshly made 4% PFA for 10 minutes, washed with PBS three times, treated with Proteinase K at 1 mg/ml for 5 minutes, fixed with 4% PFA for 5 minutes, washed with PBS three times, acetylated for 10 minutes in 1.25% triethanolamine; 0.25% acetic acid anhydride; 0.175% HCl, washed with PBS three times and equilibrated with hybridization buffer (50% formamide; 1x Denhardt’s solution; 0.25 mg/ml Baker’s yeast RNA; 0.1 mg/ml Salmon sperm DNA in 5x SSC buffer) for 2 hours at room temperature in a humidified chamber. Slides were dried and hybridization buffer containing 100 ng of DIG-labeled probe were added, covellsersipped and incubated at 70°C overnight. Post-hybridization, slides were treated as follows: rinsed in 0.2x SSC buffer for 1 hour at 72°C, washed with 0.2x SSC buffer at room temperature for 5 minutes, washed with B1 buffer (100 mM Tris HCl pH 7.5; 150 mM NaCl) for 5 minutes, equilibrated with 10% heat-inactivated normal goat serum (HINGS) in B1 buffer for 1 hour, washed with B1 buffer three times and incubated with anti-DIG antibody (Roche) at 1/5000 in 1% HINGS in B1 buffer for 3 hours in a humidified dark chamber at room temperature. For signal detection, slides were washed with B1 buffer three times, equilibrated with B3 buffer (40 mM Tris HCl pH 9.5; 40 mM NaCl; 20 mM MgCl₂) for 15 minutes, dried, developing solution (0.24 mg/ml tetramisole hydrochloride; 0.35 mg/ml NBT; 0.18 mg/ml BCIP in B3 buffer) was added, covellsersipped and kept in a dark chamber at room temperature until signal was detected. To stop the reaction, slides were incubated in ddH₂O for 20 minutes at room temperature four times and mounted with pre-warmed Glycergel (Agilent).

Three-dimensional positional analysis

Three-dimensional positional analysis was performed as previously described (Dewitz et al., 2018). Briefly, for the embryonic analysis, lumbar levels L1 - L3 of e13.5 spinal cords were analyzed by cutting 16 μm consecutive cryosections and performing immunostaining with Isl1 antibody for LMCm neurons and Hb9 antibody for LMCI neurons. Images were acquired using a Zeiss LSM800 confocal microscope. Regions of interest corresponding to each section and consisting of 4 - 8 tiles, depending on the spinal cord size were defined and then imaged with a 10x air objective. Cartesian coordinates of neurons were obtained using the imaging software IMARIS 9.1. To account for differences in spinal cord size, orientation and shape, the datasets were rotated and normalized against a standardized spinal cord whose size was determined empirically (ML: 365 μm, DV: 700 μm). To align the datasets along the RC axis (z coordinate), the section where the first Isl1⁺ LMCm neurons were observed was defined as z = 0. The rostro-caudal position of each neuron was obtained by keeping track of the order of the sections. The x, y and z coordinates were then used to digitally reconstruct the distribution of the neurons. The same method was applied to postnatal datasets (motor pools positional analysis and rabies tracing analysis) using 40 μm cryosections. The border between L4 and L5 (motor pools) or T1 and T2 (RV tracing) was defined as z = 0 and consecutive sections up to 2mm in the caudal (motor pools) or rostral (RV tracing) direction were analyzed, respectively. The size of the standardized spinal cord was adjusted to ML: 1000 μm, DV: 700 μm (motor pools) or ML: 1000 μm, DV: 500 μm (RV tracing).

Positional datasets were analyzed using custom scripts in “R project” (R Foundation for Statistical Computing, Vienna, Austria, 2005). In particular, contour and density plots were generated using the “ggplot2” package, which estimates the two-dimensional Gaussian density for the distribution of the given values. Correlation analysis was done with the “corrplot” package, which calculates the similarity between pairs of experiments using the Pearson correlation coefficient. Datasets were clustered hierarchically.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical details about experiments can be found in figure legends. Unless otherwise stated, quantifications were performed by averaging parameters of three biological replicates per genotype. Significance was determined by applying unpaired, two-tailed t test. Significance was defined as: p < 0.05 = *; p < 0.01 = **; p < 0.001 = ***. Statistical analyses were performed with GraphPad Prism v7.2.

In order to account for differences in size and shape of the spinal cords, the extent of afadin/phalloidin expression and the length of dorsal and ventral gray matter in *afadin*\(^{fl/fl}\) *Wnt1* mice were normalized against the total dorso-ventral extent of gray matter. Measurements of five sections per animal were averaged.
Supplemental Information

Afadin Signaling at the Spinal Neuroepithelium Regulates Central Canal Formation and Gait Selection

Sophie Skarlatou, Coralie Hérent, Elisa Toscano, César S. Mendes, Julien Bouvier, and Niccolò Zampieri
Figure S1
**Figure S1. Afadin elimination does not affect motor neuron development (related to Figure 1).**

A and B) Afadin expression in e13.5 control (A) and *afadin*[^β/β] (B) spinal cords.

C) Average number of medial and lateral LMC neurons (LMCm and LMCl) per 100 μm in control and *afadin*[^β/β] embryos (mean ± SEM; t-test, p > 0.05; n = 3 for each genotype).

D and E) Transverse sections showing Isl1[^+^] LMCm and Hb9[^+^] LMCl neurons in e13.5 control (D) and *afadin*[^β/β] (E) hemicords.

F and G) Transverse contour density plots of Isl1[^+^] LMCm (green) and Hb9[^+^] LMCl (red) neurons in control (F) and *afadin*[^β/β] (G) embryos.

H and I) Longitudinal contour density plots of Isl1[^+^] LMCm (green) and Hb9[^+^] LMCl (red) neurons in control (H) and *afadin*[^β/β] (I) embryos.

J) Boxplots showing medio-lateral distribution of LMCm and LMCl neurons in control, *afadin*[^β/β] and *afadin*[^β/c] (*afadin*[^β/c] dataset from Dewitz et al., 2018) embryos.

K) Average medio-lateral position of LMCm (green) and LMCl (red) neurons in control, *afadin*[^β/β] and *afadin*[^β/c] (*afadin*[^β/c] dataset from Dewitz et al., 2018) embryos.

L and M) CTB-labeled GS (Alexa-488) and TA (Alexa-555) motor neurons in control (L) and *afadin*[^β/β] (M) adult mice.

N and O) Transverse contour density plots of GS (green) and TA (red) motor neurons in control (N) and *afadin*[^β/β] (O) adult mice.

P and Q) Longitudinal contour density plots of GS (green) and TA (red) motor neurons in control (P) and *afadin*[^β/β] (Q) adult mice.

R) Average medio-lateral position of GS (green) and TA (red) motor neurons in control and *afadin*[^β/β] adult mice (mean ± SEM; t-test, p > 0.05; n = 3).
S) Average number of GS (green) and TA (red) cells labeled in control and *afadin*^fl/fl^ adult mice (mean ± SEM; t-test, p > 0.05; n = 3).

T) Correlation analysis of GS and TA neurons Cartesian coordinates in control and *afadin*^fl/fl^ adult mice. Scale bar indicates correlation values.

edio-lateral axis (n = 3).
Figure S2

Afadin Phalloidin

control

Afadin

afadin^m

Afadin

control

afadin^m

Figure S2
**Figure S2. Central canal development upon elimination of afadin in motor neuron progenitors (related to Figure 3).**

A - F) Transverse sections of e13.5 (A, B), e15.5 (C, D) and p1 (E, F) control spinal cords showing afadin (A, C, and E) and phalloidin (B, D and F) expression.

G - L) Transverse sections of e13.5 (G, H), e15.5 (I, J) and p1 (K, L) afadin$^{fl/fl}$ spinal cords showing afadin (G, I and K) and phalloidin (H, J and L) expression.

M - O) Transverse sections of e14.5 (M), e16.5 (N) and e17.5 (O) control spinal cords showing afadin expression. Arrow points to the developing central canal.

P - R) Transverse sections of e14.5 (P), e16.5 (Q) and e17.5 (R) afadin$^{fl/fl}$ spinal cords showing afadin expression. Arrows point to the developing central canals.

lateral axis (n = 3).
Figure S3. Afadin has a general role in regulating the organization of the midline (related to Figure 4).

A and B) Transverse sections of e14.5 control (A) and afaf/fl Wnt1 (B) spinal cords. Scale bar represents 100 μm.

C and D) Afadin expression in the developing central canal area of the images in A (C) and B (D). Bars represent extent of afadin expression.

E and F) Nissl staining in the dorsal midline area of the images in A (E) and B (F). Dashed lines delineate the neuroepithelium and the border between the ventral grey and white matter.
Figure S4

A  TAG-1  Afadin  C  TAG-1  Afadin
control  e13.5  e15.5  e13.5  e15.5

afadin<sup>ww</sup>  ROBO3  Afadin  D  ROBO3  Afadin
control  e13.5  e15.5  e13.5  e15.5

afadin<sup>ww</sup>  ephrin B3  Rhodamine B-Dextran  F  Rhodamine B-Dextran  G
control  e13.5  afadin<sup>ww</sup>  e15.5

Figure S4
Figure S4. Analysis of commissural axon organization in afadin \( ^{β/β} \) mice (related to Figure 5).

A) Transverse sections of e13.5 control and afadin \( ^{β/β} \) spinal cords showing TAG-1 (left) and afadin (right) expression. Arrow points to the pMN.

B) Transverse sections of e13.5 control and afadin \( ^{β/β} \) spinal cords showing ROBO3 (left) and afadin (right) expression. Arrow points to the pMN.

C) Transverse sections of e15.5 control and afadin \( ^{β/β} \) spinal cords and higher magnification of the central canal area showing TAG-1 (left) and afadin (right) expression. Arrows point to the central canals.

D) Transverse sections of e15.5 control and afadin \( ^{β/β} \) spinal cords and higher magnification of the central canal area showing ROBO3 (left) and afadin (right) expression. Arrows point to the central canals.

E) Left panel: Ephrin B3 mRNA expression in a p7 af \( ^{β/β} \) ChAT spinal cord. Right panel: Magnification of the central canal area. Central canal is delineated by a dashed line.

F) Transverse section of an e15.5 control hemicord (left) and higher magnification of central canal area (right) showing tracing of spinal interneurons by rhodamine dextran.

G) Transverse section of an e15.5 afadin \( ^{β/β} \) hemicord (left) and higher magnification of central canal area (right) showing tracing of spinal interneurons by rhodamine dextran.
Figure S5
**Figure S5. Premotor connectivity maps in control and afadin<sup>0/0</sup> mice (related to Figure 6).**

A and B) Digital reconstruction of premotor interneurons positions in two control (A) and afadin<sup>0/0</sup> (B) mice.

C) Dorso-ventral density analysis of contralateral rabies-labeled premotor interneurons in control and afadin<sup>0/0</sup> mice (n = 3; each trace represents one animal).

D) Correlation analysis of rabies-labeled premotor interneuron cartesian coordinates in control and afadin<sup>0/0</sup> mice. The scale indicates correlation values.

E and F) Digital reconstruction of rabies<sup>+</sup>/Lbx1<sup>+</sup> premotor interneuron positions in three control (E) and afadin<sup>0/0</sup> (F) mice.
Figure S6. FoxD3\textsuperscript{+} premotor interneuron connectivity is unaffected in afadin\textsuperscript{ββ} mice (related to Figure 7).

A) Left panel: Digital reconstruction of total contralateral premotor interneurons mice (n = 3). Right panel: Digital reconstruction of rabies\textsuperscript{+}/Lbx1\textsuperscript{+} subset in afadin\textsuperscript{ββ} mice (n = 3).

B) Medio-lateral density analysis in the contralateral side of total (black) and rabies\textsuperscript{+}/Lbx1\textsuperscript{+} (magenta) premotor interneurons in afadin\textsuperscript{ββ} mice (n = 3 for each).

C and D) Ipsilateral transverse sections of a control (C) and an afadin\textsuperscript{ββ} (D) spinal cord showing rabies-labeled premotor interneurons and FoxD3 expression. Arrows are pointing at double positive cells magnified in the insets.

E and F) Digital reconstruction of rabies\textsuperscript{+}/FoxD3\textsuperscript{+} premotor interneuron positions in control (E) and afadin\textsuperscript{ββ} (F) mice (n = 3).

G) Percentage of contralateral rabies\textsuperscript{+}/FoxD3\textsuperscript{+} premotor interneurons (mean ± SEM; t-test, p > 0.05; n = 3).

H) Medio-lateral density analysis of rabies\textsuperscript{+}/FoxD3\textsuperscript{+} premotor interneurons in control and afadin\textsuperscript{ββ} mice (n = 3).

I) Representative images of a premotor interneuron in the dorsal contralateral quadrant of a control and an afadin\textsuperscript{ββ} spinal cord stained for Lhx1 and FoxD3.
**Figure S7. Positional organization of interneuron subtypes is not perturbed in afadin^{fl/fl} mice (related to Figure 7).**

A and B) Digital reconstruction of ChAT$^+$ interneuron positions in control (A) and *afadin^{fl/fl}* (B) mice (n = 3).

C) Boxplots showing distribution of ChAT$^+$ interneurons in control and *afadin^{fl/fl}* mice along the dorso-ventral axis (n = 3).

D) Boxplots showing distribution of ChAT$^+$ interneurons in control and *afadin^{fl/fl}* mice along the medio-lateral axis (n = 3).

E and F) Digital reconstruction of Chx10$^+$ interneuron positions in control (E) and *afadin^{fl/fl}* (F) mice (n = 3).

G) Boxplots showing distribution of Chx10$^+$ interneurons in control and *afadin^{fl/fl}* mice along the dorso-ventral axis (n = 3).

H) Boxplots showing distribution of Chx10$^+$ interneurons in control and *afadin^{fl/fl}* mice along the medio-lateral axis (n = 3).
Supplemental Table 1. Related to Figure 6 and 7.

Number of neurons labeled in each rabies experiment performed (The ChAT+ datasets have also been used for total premotor neuron analysis in Figures 6A-J and S5A-D).

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