Reviewed Preprint
v1 • April 23, 2026
Not revised

Reviewed Preprint
v2 • October 9, 2026
Revised by authors

Neurotrophin-3 produced by motor neurons non-cell autonomously regulates the development of pre-motor interneurons in the developing spinal cord

For correspondence:
frederic.clotman@uclouvain.be

* these two authors equally contributed to the work

Centre for Biomedical Research in Mental Health (CIBERSAM), Madrid, Spain; Neuropsychopharmacology & Psychobiology Research Group, Department of Psychology, University of Cadiz, Cádiz, Spain; Biomedical Research and Innovation Institute of Cádiz (INiBICA), Cádiz, Spain.

Competing interests: No competing interests declared

Funding: See page 22

Reviewing editor: Paschalis Kratsios, University of Chicago, United States

© 2026, Angla-Navarro et al. This article is distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use and redistribution provided that the original author and source are credited.

Andrea Angla-Navarro[1,2,*], Ana Dominguez Bajo[1,2,*], Mathilde Toch[2], Cédric Francius[2], Maria Hidalgo-Figueroa[2,#], Jingwen Zhang[1], Mélina Finet[1], Olivier Schakman[3], Manon Martin[4], Xiuqian Mu[5], René Rezsohazy[1], Françoise Gofflot[1], Frédéric Clotman[1,2]

[1]Université catholique de Louvain, Louvain Institute of Biomolecular Science and Technology, LIBST, Animal Molecular and Cellular Biology (AMCB), Louvain la Neuve, Belgium • [2]Université catholique de Louvain, Institute of Neuroscience, IoNS, Laboratory of Neural Differentiation (NEDI), Woluwe-Saint-Lambert, Belgium • [3]Université catholique de Louvain, Institute of Neuroscience (IoNS), Behavioral analysis platform, Brussels, Belgium • [4]Université catholique de Louvain, Louvain Institute of Biomolecular Science and Technology (LIBST), Louvain la Neuve, Belgium • [5]University at Buffalo, Department of Ophthalmology/Ross Eye Institute, Jacobs School of Medicine and Biomedical Sciences, Buffalo, United States

eLife Assessment

This is a **useful** study that seeks to elucidate the molecular mechanisms underlying spinal circuit assembly. The authors demonstrate that Onecut transcription factors in spinal motor neurons control the development of pre-motor interneurons in a non-cell autonomous manner. However, certain conclusions are **incompletely** supported by the data at hand. The work will be of broad interest to cell and developmental biologists.

https://doi.org/10.7554/eLife.110788.2.sa4

Abstract

The development of multicellular organisms requires proper interplays between cell-autonomous genetic programs controlled by combinations of transcription factors that regulate the differentiation of distinct cell populations and non-cell autonomous processes that coordinate the proliferation, the fate, the survival, the respective location, and the proper interactions of these populations. During the development of the nervous system, non-cell autonomous mechanisms determine neuronal fate, survival, distribution, axon guidance, and connectivity. Although similar processes are suggested to be at work in the formation of spinal motor circuits, the molecular mechanisms involved remain mostly elusive. Here, we provide evidence that the Onecut transcription factors regulate a non-cell autonomous mechanism that modulate pre-motor interneuron development. We show that conditional inactivation of the Onecut factors in spinal motor neurons affects the differentiation and the positioning of pre-motor interneuron populations. We identify that Neurotrophin-3 produced by motor neurons under the control of the Onecut factors non-cell autonomously regulate the production and the distribution of pre-motor interneuron populations. Thus, we elucidated one of the non-cell autonomous mechanisms that coordinate the formation of the spinal motor circuits.

Introduction

The development of multicellular organisms requires the coordinated production and positioning of a constellation of specialized cells exerting distinct but complementary functions. Specialization of stem or progenitor cells into different cell types depends on the activation of distinct cell-autonomous genetic programs that are controlled by time- and space-regulated combinations of transcription factors (Allan and Thor, 2015; Sagner, 2024). However, this intrinsic regulation additionally necessitates extrinsic mechanisms that coordinate the proliferation, the fate, the survival, the respective location, and the proper interactions of these different cell populations (Czesnick and Lenhard, 2015; Surya and Sarinay-Cenik, 2022; Williamson, 2023). These non-cell autonomous processes, which rely on signals coming from the extracellular environment including secreted molecules, cell-cell interaction through membrane receptors, changes in electrical activity or mechanical forces (see for example (Choi et al., 2020; Guemez-Gamboa et al., 2014; Lim et al., 2018; Pillai and Franze, 2024; Welniarz et al., 2017)), have been less extensively studied. Nevertheless, the emergent picture reveals a dynamic landscape where signaling pathways, diffusible factors, cell activity and mechanical forces traverse cell membranes, enabling cells to communicate, cooperate and adapt, to generate functional multicellular tissues or organs.

During nervous system development, non-cell autonomous mechanisms determine neuronal fate, survival, distribution, axon guidance, and connectivity. For example, Cajal-Retzius (CR) cells, a transient class of neurons that populate the mantle zone of the mammalian cerebral cortex very early during brain development, regulate the respective size of different cortical areas (Barber et al., 2015), critically control the laminar organization of the neocortex through the secretion of Reelin (reviewed in (Jossin, 2020)) and of Nectin1 (Gil-Sanz et al., 2013), and their activity alters survival and connectivity of pyramidal neurons (Riva et al., 2019). Inversely, CR cell migration is controlled by an extracellular contingent of the homeoprotein Pax6 secreted by cortical progenitors (Kaddour et al., 2020). The proliferation, survival and radial growth of cortical progenitors and neurons are regulated by neurons that originate outside of the neocortex (Causeret et al., 2011; Teissier et al., 2010). Reciprocally, the identity, connectivity and survival of cortical interneurons (INs), which are born in subcortical ganglionic eminences and colonize the neocortex during development, is determined by the type of cortical projection neurons they approach (Lim et al., 2018; Wester et al., 2019), and their number and organization in the marginal zone are controlled by non-muscle myosin II heavy chain-mediated regulation of radial glial cell complexity and endfoot position (D'Arcy et al., 2023). Among cortical neurons, the transcription factor Foxp2 produced in layers 5 and 6 of the cerebral cortex regulate in a non-cell autonomous manner gene expression in projection neurons and the number of cortical INs (Co et al., 2020). The distribution of cortical INs is also modulated by extrinsic signaling. The proto-cadherin PCDH19 non-cell autonomously affects cell migration in medial ganglionic eminence explants (Pancho et al., 2022), and netrin signaling determines the migration of cortical INs (Yamagishi et al., 2020). In addition, axonal growth and neurite formation are modulated by extrinsic processes. Cunningham and colleagues (Cunningham et al., 2022) demonstrated that the complete genetic removal of the c-Jun N-terminal kinase gene, which is expressed in striatal, guidepost and corridor cells, causes the inability of thalamocortical axons to cross the diencephalon-telencephalon boundary, causing axonal misrouting to other cerebral areas. The netrin receptor DCC controls midline crossing of the cortico-spinal tract in a non-cell autonomous manner in mice, but not in humans (Welniarz et al., 2017), whereas lumican, an extracellular proteoglycan secreted by corticospinal lateral neuronal populations, extrinsically suppresses cervical collateralization by multiple corticospinal medial subpopulations (Itoh et al., 2023). Inversely, the ascending axonal growth of rapidly adapting mechanoreceptors to the brainstem is modulated by roof plate radial glial-like cells (Kridsada et al., 2018). Neuronal activity also triggers non-cell autonomous processes. Activity-dependent BDNF release regulates the Glutamate-GABA neurotransmitter switching in a non-cell autonomous manner in Xenopus (Guemez-Gamboa et al., 2014), and the identity and position of dorsal root ganglia neurons in mice (Wright and Ribera, 2010). In zebrafish, voltage-gated Na+ channels expressed in specific motor neuron (MN)

subtypes regulate axonal projections of other MNs that do not produce them (Pineda et al., 2006), and in the mouse GluR1-dependant activity regulates MN neurite formation and connectivity in a cell autonomous and a non-cell autonomous manner (Zhang et al., 2008). Finally, terminal maturation of neural circuit is coordinated by extrinsic cues. For example, critical periods of heightened IN plasticity are defined by multiple non-cell autonomous signals, such as brain-derived neurotrophic factor (BDNF), the synapse-regulating SPARCL1 glycoprotein, the homeoprotein OTX2 or extracellular matrix components, produced by cortical or extra-cortical sources (Bernard et al., 2016; Gibel-Russo et al., 2022; Vincent et al., 2021).

Furthermore, different pieces of published data suggest that similar coordination mechanisms are also at work in the developing spinal cord. Body movements are regulated by neural circuits located in ventral regions of the spinal cord and constituted of MNs, which directly innervate skeletal muscles, and of multiple populations of pre-motor INs, the integrated actions of which control the activity of the MNs (Cote et al., 2018). Some aspects of spinal motor circuit development are reported to rely on non-cell autonomous mechanisms. Differentiating MNs modulate the entry of MN progenitors into differentiation through a regulation of the Notch pathway involving the six-transmembrane protein glycerophosphodiester phosphodiesterase 2 (GDE2) and disinhibition of the ADAM protease by release of the RECK activator from the MN membrane (Park et al., 2013; Sabharwal et al., 2011). During V2 IN differentiation, the transcription factor GATA2, which is present in progenitors and in differentiating V2 cells, regulate in a non-cell autonomous manner the proliferation of neural progenitors through an unknown mechanism (El Wakil et al., 2006). In zebrafish, early-born primary MN determine the GABAergic phenotype of Kolmer–Agduhr INs (Seredick et al., 2014). Constitutive inactivation of *Islet-1* (*Isl1*), a transcription factor present in MNs and in dorsal dI3 INs, alters the development of V1 INs (Pfaff et al., 1996). Similarly, the transcriptional regulator Bhlhb5 influences the generation of INs surrounding its distribution domain (Skaggs et al., 2011), and inactivation of WT1 from dI6 INs changes the composition in V0 and in V2a INs (Schnerwitzki et al., 2018). Furthermore, the survival of spinal IN populations is controlled by extrinsic cues, as their apoptosis is differentially regulated by protocadherin-gamma adhesion molecules (Prasad et al., 2008). Taken together, these observations demonstrate that, in the nervous system, non-cell autonomous mechanisms regulate neuronal fate, survival, distribution, and connectivity. However, how much similar processes contribute to coordinate the development of neuronal populations forming the spinal motor circuits remains unknown.

Onecut (OC) transcription factors, including Onecut-1 (OC-1), OC-2 and OC-3, are homeodomain-containing transcriptional regulators that control multiple aspects of motor circuit development, including MN or IN differentiation or survival (Francius and Clotman, 2010, 2014; Roy et al., 2012; Stam et al., 2012; Toch and Clotman, 2019; Toch et al., 2020), IN distribution (Harris et al., 2019; Kabayiza et al., 2017), and formation of the neuromuscular junctions (Audouard et al., 2012). Here, we provide evidence that the OC factors regulate in spinal MN a non-cell autonomous mechanism that modulate pre-motor IN development. We identify candidate factors potentially involved in this process, and we demonstrate that Neurotrophin-3 (Ntf3) produced by MN contributes to the regulation of IN differentiation and distribution, and thereby coordinate proper formation of spinal motor circuits.

Results

OC factors regulate in spinal MNs a non-cell autonomous mechanism that modulates pre-motor IN development

The OC transcription factors are produced in multiple spinal neuronal populations including MNs (Francius and Clotman, 2010; Francius et al., 2013; Roy et al., 2012) and ventral (Francius et al., 2013; Harris et al., 2019; Stam et al., 2012; Toch et al., 2020) or dorsal INs (Kabayiza et al., 2017). However, OC are not present in all the cells of each population but are produced in specific subsets (Francius et al., 2013; Harris et al., 2019; Stam et al., 2012). In V1 INs, OC are detected in Renshaw cells (RC) but in none of the other V1 components (Francius et al., 2013;

Stam et al., 2012). In the course of our studies investigating the function of the OC proteins during spinal cord development in constitutive *Oc1/Oc2* double-mutant embryos (Harris et al., 2019; Stam et al., 2012; Toch et al., 2020), which additionally lack OC-3 in the spinal cord (Kabayiza et al., 2017; Roy et al., 2012), we noticed alterations in the differentiation of non-RC V1 INs (Figure 1A-C). In particular, a subset of Foxp2+ V1 INs containing the MafA transcriptional regulator increased in cell number and expanded in an abnormal ventro-medial location as compared to control embryos at embryonic day (e)11.5 (Figure 1A, C). Similarly, V1 INs abnormally producing Prdm8 (Francius et al., 2013) and Otp were observed in the same ventro-medial position at e12.5 (Figure B-C). These observations suggested that OC factors may control in the developing spinal cord a non-cell autonomous mechanism regulating the differentiation of V1 INs.

Given that OC proteins are critical for proper differentiation of the spinal MNs and expression of *Isl1* in these cells (Roy et al., 2012), that IN alterations have been reported in embryos wherein MN development is strongly affected by the absence of Isl1 (Pfaff et al., 1996), and that MNs exert non-cell autonomous regulation of the differentiation of surrounding cells (Park et al., 2013; Sabharwal et al., 2011), we hypothesized that OC factors may control this non-cell autonomous mechanism by acting in spinal MNs. To assess the possible contribution of MNs to this mechanism, we first investigated the development of pre-motor IN populations after genetic ablation of spinal MNs. Embryos wherein diphteria toxin subunit A (DTA) is produced specifically in MN progenitors upon *Olig2-Cre* activation (Dessaud et al., 2007) showed a >90% reduction in the number of differentiating MN at e11.5 (Figure 1D,F), without alteration of the pMN progenitor domain (Supplemental Figure S1). In these embryos, the number of V2b INs labelled for Gata2 was increased at brachial levels of the spinal cord (Figure 1D-E,J). In contrast, the amount of V0v characterized by the presence of Evx1 and of V1 assessed by the production of Foxd3 (Francius et al., 2013) was significantly reduced at thoracic levels (Figure 1D-E,G-H), while the production of V2a INs was not changed (Figure 1D-E,I). The V3 INs were not investigated, as the *Olig2-Cre* allele also drives recombination, and therefore DTA production, in this population (Chen et al., 2011; Debrulle et al., 2020). Distribution of the pre-motor IN population was not analyzed in detail, as the ablation of MNs resulted in a global ventral displacement of all the IN populations (Figure 1D-E). Nevertheless, these observations suggest that MNs modulate the development of surrounding pre-motor IN populations in the developing spinal cord.

To confirm this interpretation and the possible implication of OC factors in this process, we studied the phenotype of ventral spinal INs after combined conditional inactivation of *Oc1* and *Oc2* in spinal MNs at e12.5 (Figure 2) and at e14.5 (Supplemental Figure S2)(Toch et al., 2020). Absence of OC factors does not alter the number of MNs(Roy et al., 2012). The total number of V1 INs, assessed here by the presence of Foxp2 (Francius et al., 2013), of V2a and of V2b INs was not modified when OC factors were absent from MNs (Figure 2A-F; Supplemental Figure S2A-C). In contrast, the number of V2c INs, which contain Sox1 and are located outside of the ventricular zone in the vicinity of the MNs (Panayi et al., 2010), was drastically reduced in the conditional *Oc* double-mutant embryos (Figure 2G-H; Supplemental Figure S2D), without any increase in spinal cord cell death (Roy et al., 2012). Thus, except for the V2c population, absence of OC factors from the MNs does not alter the global number of ventral INs, consistent with previous observations in the constitutive OC mutants wherein the size of the IN populations was not altered (Harris et al., 2019; Toch et al., 2020).

In contrast, the distribution of ventral IN populations on the transverse plane of the spinal cord was systematically modified, as observed in constitutive OC mutants (Harris et al., 2019; Kabayiza et al., 2017; Toch et al., 2020). In particular, the V1 population was slightly but significantly dorsally mislocated at thoracic levels and ventrally displaced at brachial and lumbar levels in the conditional OC double-mutant embryos at e12.5, as compared to control embryos (Figure 2I). At e14.5, V1 INs settled slightly more dorsal at brachial and lumbar levels while they migrated more ventrally in the control embryos (Supplemental Figure S2E). At e12.5, V2a INs were more dorso-medially located at brachial and thoracic levels but remained more medial at

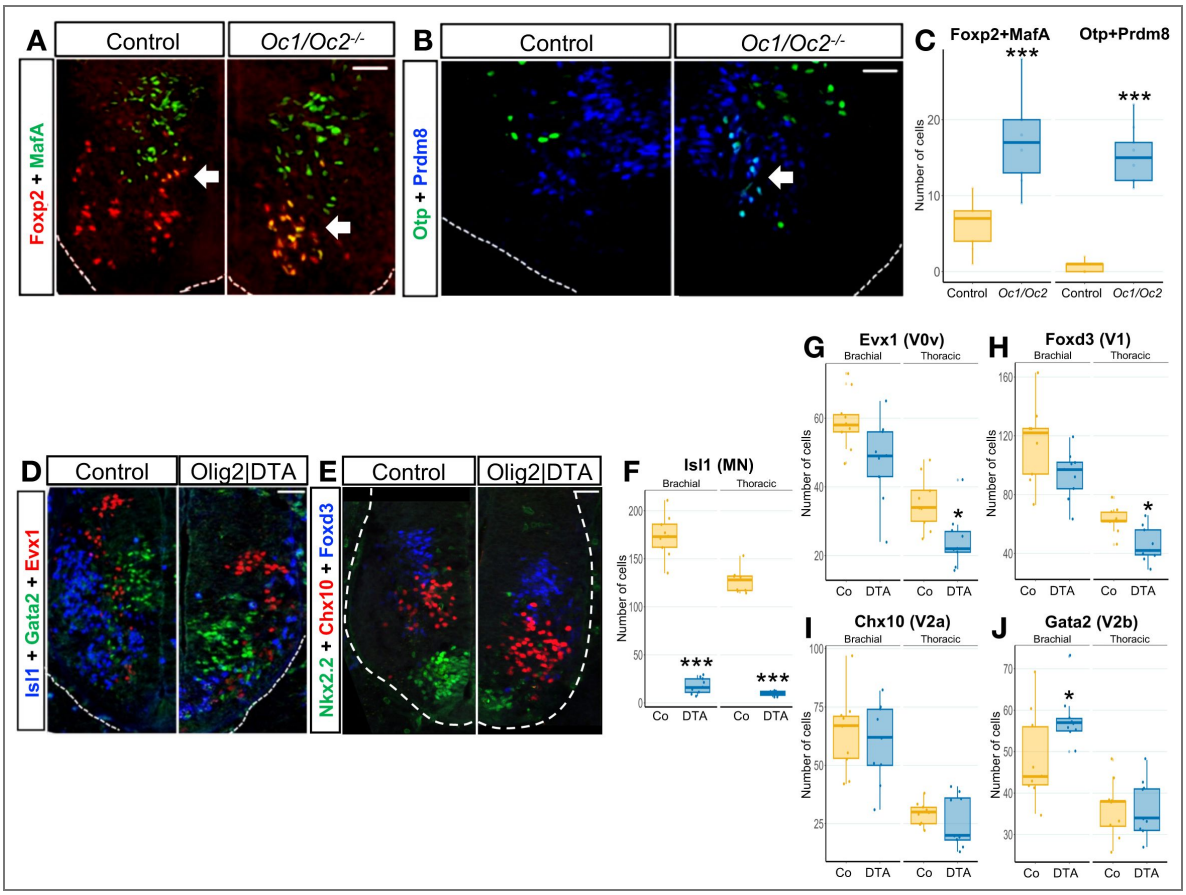


Figure 1. Spinal interneuron development is altered in Onecut constitutive mutants and in embryos depleted in spinal motor neurons.

(A-C) In constitutive *Oc1/Oc2* double-mutant embryos, which additionally lack OC-3 in the spinal cord, a subset of Foxp2+ V1 INs containing the MafA transcriptional regulator (yellow) increased in cell number and expanded in an abnormal ventro-medial location as compared to control embryos at embryonic day (e)11.5 (arrows; control left hemicord, mutant right hemicord; thoracic levels). Similarly, V1 INs abnormally producing Prdm8 and Otp (cyan) were observed in the same ventro-medial position at e12.5 (arrow). (D-J) After genetic ablation of ~90% of spinal MNs, the number of V0v INs characterized by the presence of Evx1, and of V1 INs assessed by the production of Foxd3, was significantly reduced at thoracic levels of the spinal cord. In contrast, the number of V2b INs, which contain Gata3, was increased at brachial levels, whereas V2a INs identified by the presence of Chx10 were unchanged. Scale bars=100μm. n=3; *=adjusted p value < 0.05; ***=adjusted p value < 0.001

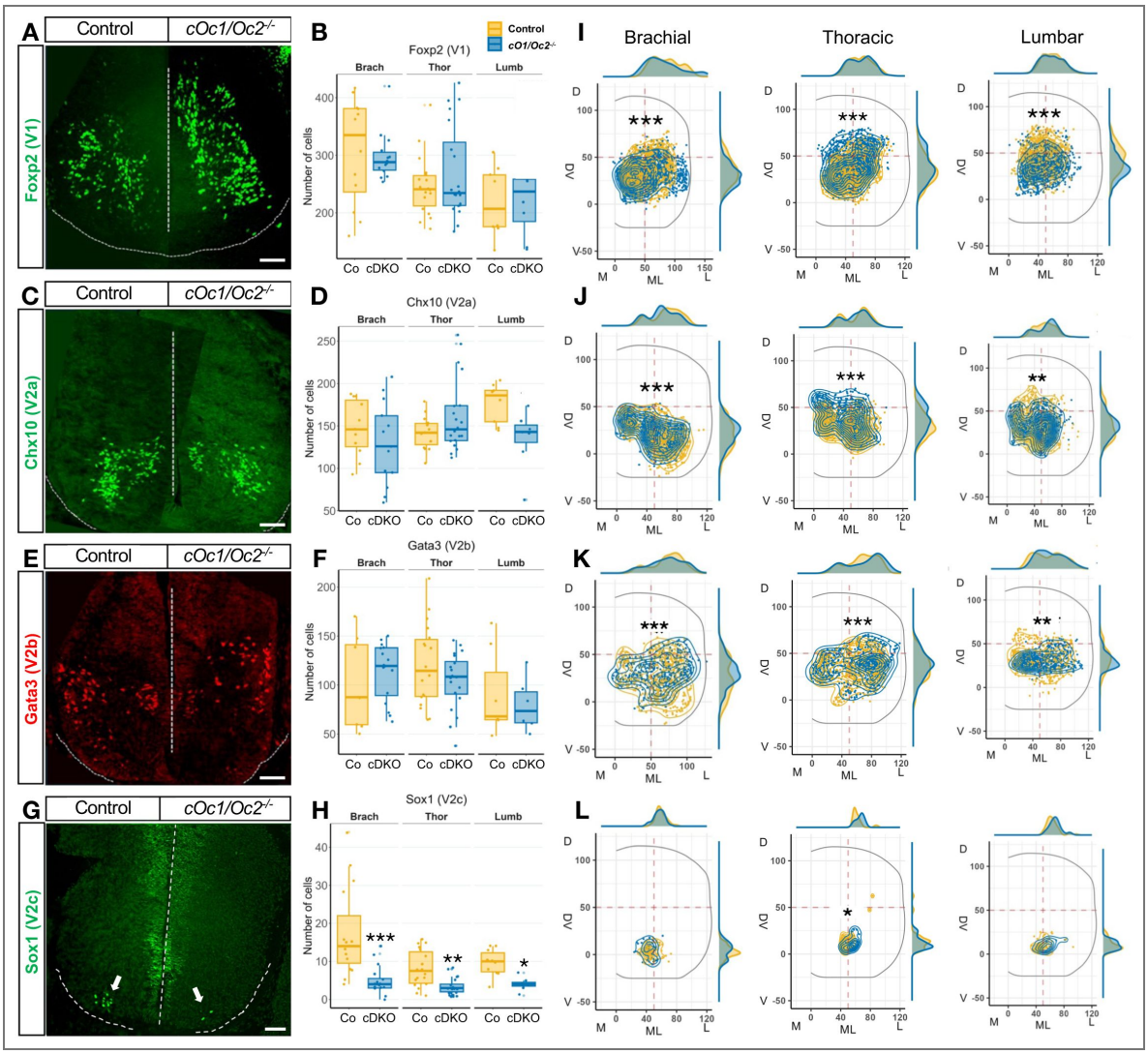


Figure 2. Spinal interneuron number and positioning is altered when Onecut factors are absent from the spinal motor neurons.

(A-L) In MN conditional *Oc1/Oc2* double-mutant embryos (cDKO) at e12.5, the number of V2c INs identified by the presence of Sox1 outside of the ventricular zone was significantly reduced at all levels of the spinal cord. Furthermore, the positioning of the V1 (Foxp2), V2a (Chx10), V2b (Gata3) and V2c INs on the transversal plane of the spinal cord was significantly altered. Labelings are shown at thoracic levels of the spinal cord. Scale bars=100μm. ML: medio-lateral axis; DV: dorso-ventral axis. n=3; *=adjusted p value < 0.05; **=adjusted p value < 0.01; ***=adjusted p value < 0.001

lumbar levels at e12.5 (Figure 2J), whereas at e14.5 they settled more dorsal at brachial and thoracic levels and more ventral at lumbar levels (Supplemental Figure S2F). In contrast, V2b INs were less scattered than in the control embryos, concentrating in a more medial position along the dorso-ventral axis of the spinal cord, and more laterally at thoracic and lumbar levels at e12.5 (Figure 2K) and at e14.5 (Supplemental Figure S2G). Finally, although the population was smaller than in controls, some V2c INs were located more laterally at thoracic levels at e12.5 (Figure 2L) but retained this position while control V2c migrated more laterally at e14.5 (Supplemental Figure S2H). Taken together, these observations indicate that OC factors regulate a non-cell autonomous mechanism in MNs that significantly impact the distribution of pre-motor IN populations in the developing ventral spinal cord.

OC factors moderate the expression of *Neurotrophin-3* (*Ntf3*) in spinal MNs

To identify genes downstream of OC factors in spinal MNs that could contribute to this non-cell autonomous mechanism, we conducted a bulk RNA-sequencing comparison of the transcriptome of control and of OC-deficient MNs at e10.5 (Figure 3A; GEO repository, accession number: GSE141949)(Toch et al., 2020). This transcriptomic analysis unveiled changes in the expression levels of genes coding for membrane proteins or secreted factors, potentially involved in non-cell autonomous processes (Figure 3B-C; Supplemental Figure S3). Among these, the expression of *Frem1* and *2* (Fras1-related extracellular matrix proteins), *Fat3* (FAT atypical cadherin 3), *Sema3d* encoding a secreted signaling protein of the Semaphorin III family and the neurotrophin receptor *Gfra3* (GDNF family receptor alpha 3) was downregulated in the absence of OC factors. In contrast, expression of *Tac1* (Tachykinin precursor 1) encoding four products of the tachykinin peptide hormone family (substance P, neurokinin A, and neuropeptides K and gamma) that can act as neurotransmitters, *Smim18* (Small integral membrane protein 18), *Cbln2* coding for the secreted glycoprotein Cerebellin-2 and the neurotrophic factor *Ntf3* was increased (Figure 3D and data not shown). Possible impact of MN expression of these genes on the development of pre-motor INs was assessed by overexpression or downregulation after chicken embryonic spinal cord electroporation. However, only *Ntf3* overexpression did mildly alter ventral IN development (Supplemental Figure S4 and data not shown). To assess the expression pattern of *Ntf3* in the developing spinal cord and confirm increased expression in the absence of OC factors, we conducted *in situ* hybridization for *Ntf3* on control or conditional OC double-mutant spinal cord sections at e12.5 (Figure 3D). In control embryos, a weak signal for *Ntf3* was detected in the ventral spinal cord at the location of the motor columns (Figure 3D), consistent with the reported expression of *Ntf3* specific to spinal MNs that decreases between e10.5 and e13.5(Buck et al., 2000; Henderson et al., 1993; Usui et al., 2012). In conditional mutant embryos, the expression level of *Ntf3* was increased and became readily observable but remained restricted to the MNs of the spinal cord (Figure 3D). This indicates that Ntf3 is normally produced by MNs in the developing spinal cord and that OC factors moderate *Ntf3* expression levels in these cells. To determine which cells could respond to Ntf3 secretion by MNs, we performed immunodetection of its cognate receptor TrkC. TrkC was broadly detected in the ventral half of the spinal cord including V1, V2a and V2b interneurons, except in newly-born medial cells (Figure 3E-G), indicating that it is present at the membrane of differentiating ventral INs (Henderson et al., 1993; Usui et al., 2012). Thus, Ntf3 could participate in the non-cell autonomous control of pre-motor IN development by the spinal MNs.

Ntf3 production by MNs is required for proper development of pre-motor INs

To validate this hypothesis, we conditionally inactivated *Ntf3* in spinal MNs and analyzed the production and distribution of pre-motor IN populations. *Ntf3* inactivation did not increase spinal cord cell death (Supplemental Figure S5). In embryos carrying a MN-specific deletion of *Ntf3* at e12.5, the number of V1 and of V2a INs was significantly increased at all levels of the spinal cord (Figure 4A-D). Similarly, a significant increase in V2b was observed at thoracic levels (Figure 4E-

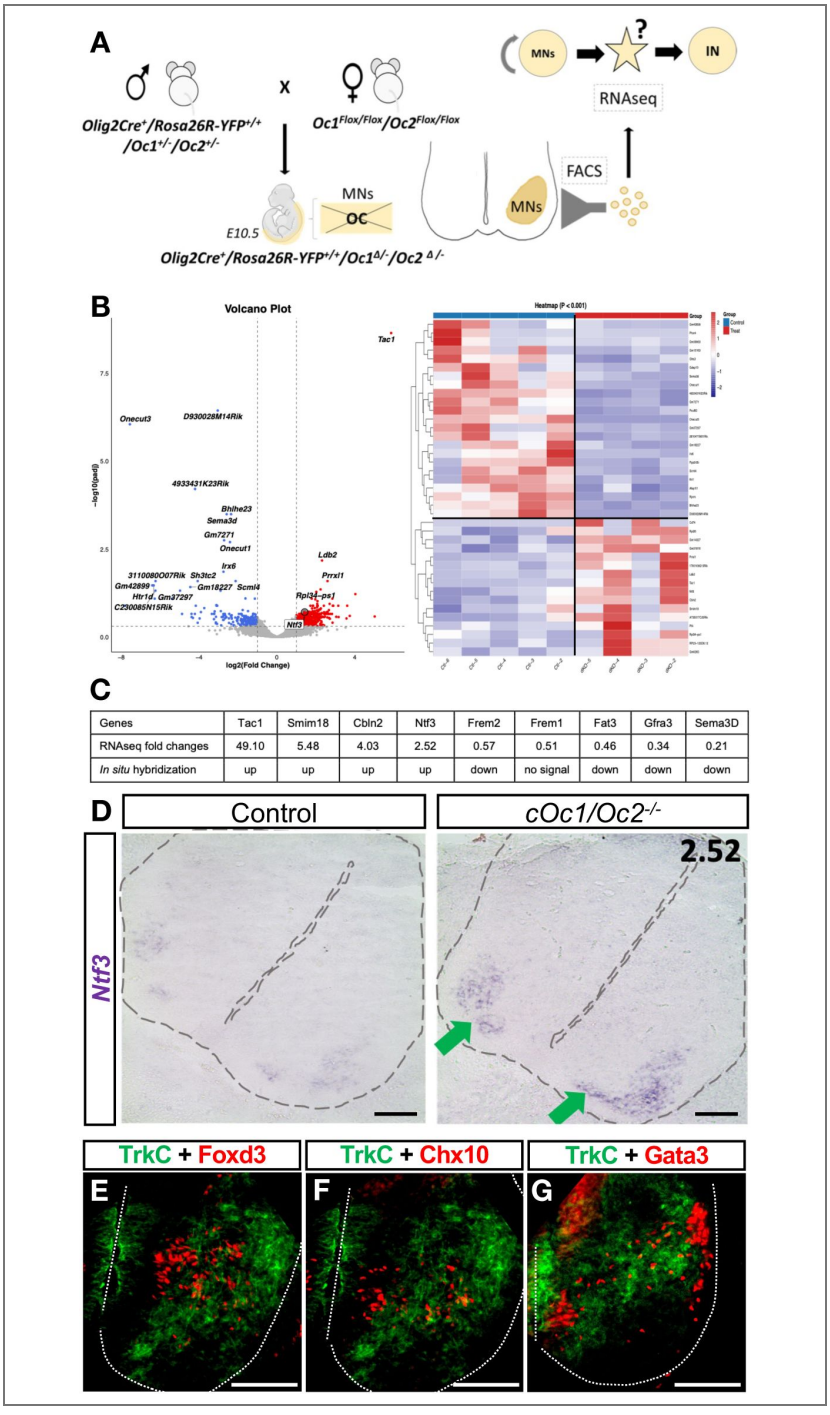


Figure 3. Ntf3 is expressed in spinal motor neurons under the control of the Onecut transcription factors.

(A) RNA-sequencing comparison of the transcriptome of control and of OC-deficient MNs at e10.5. The spinal cord of control or MN conditional *Oc1/Oc2* double-mutant embryos was isolated, dissociated, YFP-positive MNs were FACS-sorted, RNA was extracted and compared by bulk RNA sequencing. **(B)** Volcano plot or heat-map of the most down- or up-regulated genes in spinal MN deficient in Onecut factors. *Ntf3* is highlighted in the Volcano plot (see GEO repository, accession number GSE141949 for more details). **(C)** Transcriptomic analysis unveiled changes in the expression levels of selected genes encoding membrane proteins or secreted factors. MN-specific expression and changes in expression levels were confirmed by *in situ* hybridization (n=5). **(D)** Expression of the neurotrophic factor *Ntf3* was specific to MNs and increased in the absence of Onecut factors (green arrows). Labelings are shown at thoracic levels of the spinal cord. **(E-G)** TrkC, the cognate receptor of Ntf3, was broadly detected in the ventral half of the spinal cord including V1 (Foxd3, E), V2a (Chx10, F) and V2b (Gata3, G) interneurons, with the exception of newly-born neurons located in medial position. Scale bars=100µm.

F), whereas V2c INs were unchanged (Figure 4G-H). At e14.5, the number of V1 was still increased at all levels (Supplemental Figure S6A), whereas V2a increase was only maintained at lumbar levels (Supplemental Figure S6B), suggesting that programmed cell death of INs that is initiated at this stage (Prasad et al., 2008) may be increased in the absence of Ntf3. Nevertheless, impaired production of Ntf3 by the MNs results in an excess of spinal pre-motor INs in the V1 and V2 populations (Supplemental Figure S6).

However, IN distribution was additionally affected in the MN-specific OC conditional mutants (Figure 2). Therefore, we also assessed their localization at e12.5 in the absence of Ntf3. At e12.5, V1 INs, which were more numerous (Figure 4A-B), located more laterally and more ventrally than in control embryos, particularly at thoracic and lumbar levels (Figure 4I). This abnormal lateral positioning was maintained at e14.5 (Supplemental Figure S6E). V2a INs remained in a more medio-dorsal position at brachial levels but clustered in the center of the V2a distribution domain at thoracic and lumbar levels at e12.5 (Figure 4J). At e14.5 at brachial levels, V2a did not migrate dorsally as observed in control embryos and located more laterally at brachial levels (Supplemental Figure S6F). In contrast, V2b INs clustered in the lateral half of the medio-lateral axis and located more dorsal than in control embryos at all levels of the spinal cord at e12.5, with some cells aberrantly migrating in a peripheral ventro-lateral position (Figure 4K). This abnormal positioning was maintained at brachial levels at e14.5 (Supplemental Figure S6G). Finally, V2c INs gathered in small, separated groups of cells in the absence of Ntf3, both at e12.5 (Figure 4L) and e14.5 (Supplemental Figure S6H). Thus, in addition to their differentiation, Ntf3 produced by the MNs control the distribution of spinal pre-motor INs in the developing spinal cord, suggesting that Ntf3 coordinates the development of neuronal populations involved in spinal motor circuit formation. To approach this question, we conducted rotarod and balance beam tests to assess coordination in the locomotor circuits built in the absence of Ntf3 from MNs. Both female and male mice deficient for Ntf3 were almost unable to cope with rotarod acceleration, and motor learning was almost absent in these mice, as compared to control littermates (Figure 5A,D). In the balance beam test, females lacking Ntf3 in spinal MNs showed an increased time to cross the beam and increased number of footslips (Figure 5B-C), which was not the case in males (Figure 5E-F). However, learning with time normalized both parameters in females after 3 weeks, as compared to controls (Figure 5B-C). These observations are consistent with the hypothesis that Ntf3 produced by MNs is required to generate locomotor circuits with properly coordinated activity.

Discussion

Neural circuits are composed of different types of neurons that interconnect to produce a properly balanced and tightly regulated activity. Cell autonomous mechanisms that control neuronal diversity and connectivity have been widely studied. In contrast, non-cell autonomous processes that coordinate the development of different neuronal types and their integration into functional networks have been less extensively investigated, especially in the spinal cord. Here, we provide evidence that Ntf3 produced in spinal MNs under the control of OC transcription factors coordinate the development of pre-motor INs and the formation of the spinal locomotor circuits.

Our observations in different experimental models highlight the contribution of MNs to IN differentiation and distribution. However, these different models are difficult to compare. MN ablation in *Olig2|DTA* embryos is incomplete, suggesting that residual Ntf3 remains secreted in the ventral spinal cord. *OC* inactivation increases *Ntf3* expression, but to what extent and where downstream signaling is increased remains experimentally out of reach. Furthermore, we obtained evidence that OC factors can travel between cells in the developing spinal cord (data not shown), as previously described for other homeoproteins (reviewed in (Di Nardo et al., 2020)), suggesting that multiple non-cell autonomous processes are at play. Yet, our observations show that disturbing Ntf3 production in spinal MNs impact pre-motor IN development.

During embryonic development, OC transcription factors are present in multiple spinal neuronal populations, including MNs (Francius and Clotman, 2010; Roy et al., 2012), and ventral (Francius et al., 2013; Harris et al., 2019; Stam et al., 2012) or dorsal (Kabayiza et al., 2017)

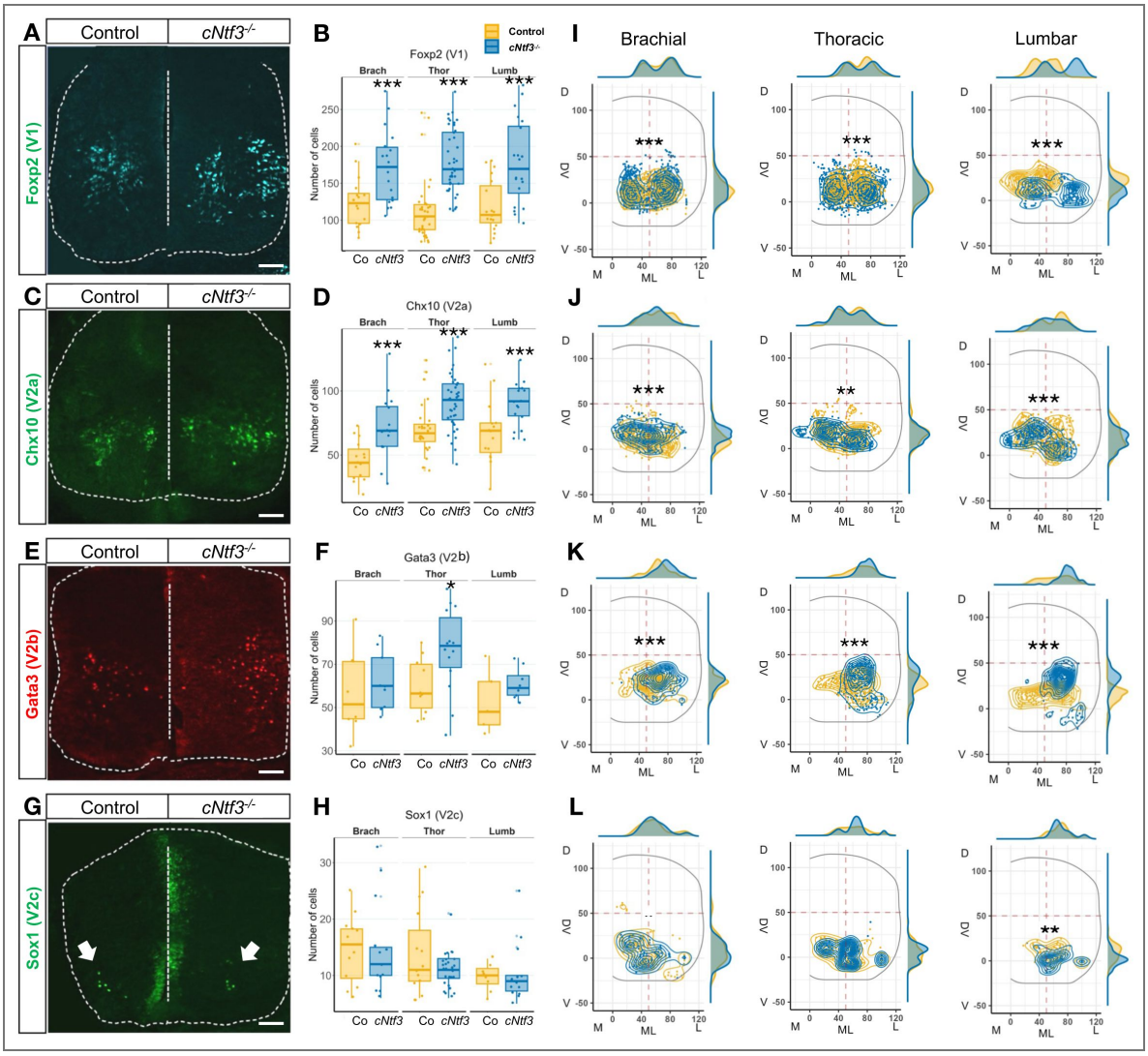


Figure 4. Spinal interneuron number and positioning is altered when Ntf3 is inactivated in the spinal motor neurons.

(A-L) In MN conditional *Ntf3* mutant embryos (*cNtf3*) at e12.5, the number of V1 (Foxp2) and of V2a (Chx10) INs was significantly increased at all levels of the spinal cord, while V2b (Gata3) only expanded at thoracic level, and V2c (Sox1, white arrows) were not altered. Furthermore, the positioning of all these IN populations on the transversal plane of the spinal cord was altered at all levels of the spinal cord. Labelings are shown at thoracic levels of the spinal cord. Scale bars=100μm. ML: medio-lateral axis; DV: dorso-ventral axis. n=3; *=adjusted p value < 0.05; **=adjusted p value < 0.01; ***=adjusted p value < 0.001

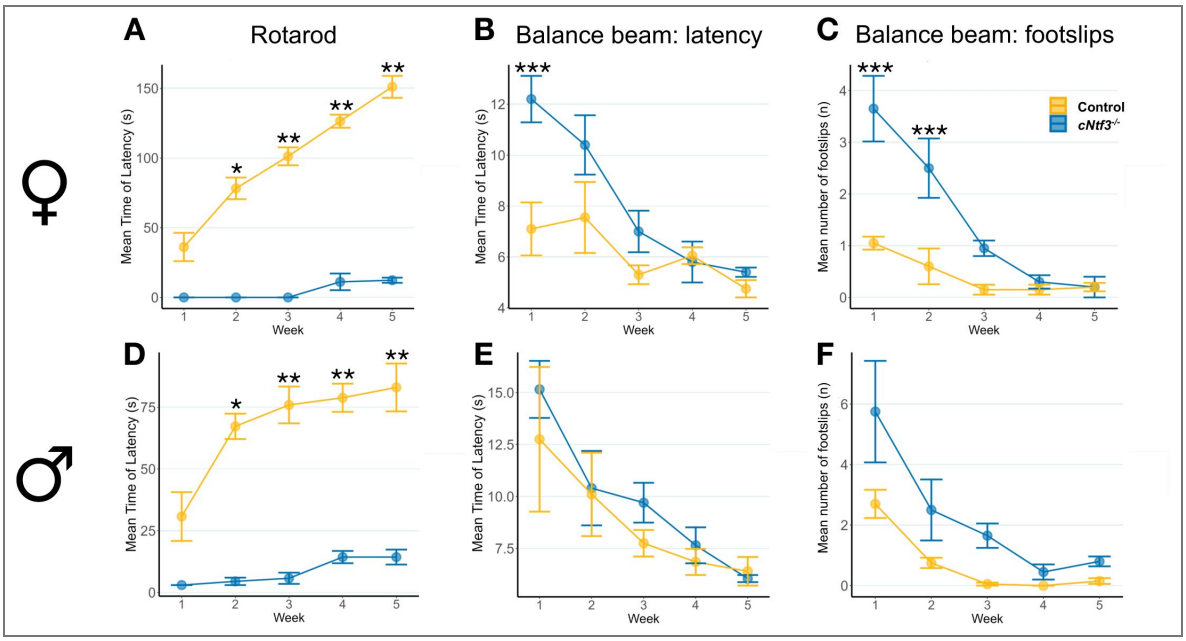


Figure 5. Locomotor coordination is altered when Ntf3 is inactivated in the spinal motor neurons.

(A-C) In female mice, while control animals demonstrated proper motor coordination and efficient motor learning evidenced by the increase in rotarod latency with time, animals carrying a conditional *Ntf3* mutation in MNs (*cNtf3*) were almost unable to cope with rotarod acceleration and initially showed increased balance beam latency and footslips, which however normalized with time. **(D-F)** In male mice, conditional *Ntf3* mutants showed motor coordination and learning defects on the rotarod but didn't demonstrate any alteration in the balance beam test. n=8 in each group; *= p value < 0.05; **= p value < 0.01; ***= p value < 0.001

INs. In this tissue, they regulate neuronal differentiation (Harris et al., 2019; Kabayiza et al., 2017; Roy et al., 2012), maintenance (Stam et al., 2012) and IN distribution (Harris et al., 2019; Kabayiza et al., 2017). Although some of these functions are very likely, at least partly, cell autonomous, involving the regulation of *Isl1* in MNs (Rhee et al., 2016; Roy et al., 2012; Toch and Clotman, 2019; Velasco et al., 2017) and of *Pou2f2* or *Nkx6.2* in INs and MNs (Harris et al., 2019; Masgutova et al., 2019; Toch et al., 2020), observations in the constitutive OC mutant embryos suggested that these factors may additionally regulate spinal IN development in a non-cell autonomous manner. Non-cell autonomous regulation of neuronal development in the embryonic spinal cord has already been reported. Murine MNs exert negative feedback on the entry of their own progenitors into differentiation (Park et al., 2013; Sabharwal et al., 2011) and seem to modulate the development of V1 INs (Pfaff et al., 1996), whereas zebrafish primary MNs determine the GABAergic phenotype of Kolmer–Agduhr INs (Seredick et al., 2014). Given that the onset of spinal MN development precedes that of INs, that MNs exert non cell-autonomous regulation of the differentiation of surrounding cells (Park et al., 2013; Pfaff et al., 1996; Sabharwal et al., 2011), that IN alterations have been reported in embryos wherein MN differentiation is strongly affected by the absence of Isl1 (Pfaff et al., 1996), and that OC proteins are critical for proper differentiation of the spinal MNs and for the expression of *Isl1* in these cells (Roy et al., 2012), we hypothesized that OC factors may regulate the observed non-cell autonomous mechanism by acting in spinal MNs. Our observations suggest that MNs control the differentiation and the positioning of the spinal pre-motor INs.

Severe immunotoxic ablation of MNs in an organotypic culture model of embryonic rat spinal cord resulted in a decreased number of inhibitory INs, which was rescued by Ntf3. In the same model, anti-serum blockade of Ntf3 activity was sufficient to more severely deplete inhibitory INs, partly through apoptosis (Bechade et al., 2002). In contrast, conditional *Ntf3* deletion in the mouse rather increased the number of V1 or V2b inhibitory INs, and of V2a excitatory neurons. Critical differences between our *in vivo* model and the highly apoptotic-prone organotypic culture model likely contribute to these discrepancies. Nevertheless, these observations support the contribution of spinal MNs to the control of IN development and the implication of Ntf3 in this process. Opposite results were obtained by other researchers using mouse models lacking almost all MNs due to the elimination of skeletal muscles at late stages of embryonic development (E18.5), where the number of spinal INs remained unaffected (Grieshammer et al., 1998; Kablar and Rudnicki, 1999). This suggests that a critical window of *Ntf3* expression at early stages of spinal development could be critical for proper production of spinal INs. Increased production of specific IN populations in the absence of Ntf3 from MNs, combined with the decrease in V2c observed in the OC mutant embryos wherein *Ntf3* expression is increased, suggest that Ntf3 does not regulate the survival of spinal pre-motor INs, in contrast to sensory neurons (ElShamy and Ernfors, 1996a, b; Liebl et al., 1997), but could rather directly contribute to their differentiation. Consistently, Ntf3 has been shown to cooperate with the morphogen Shh to promote neuronal differentiation (Dutton et al., 1999). In addition, our observations suggest that Ntf3 produced by spinal MNs regulate the migration of pre-motor INs, as the distribution of these cells was affected in embryos lacking Ntf3 from MNs. The characterization of functionally distinct IN subpopulations unveiled a strong correlation between the distribution of each IN subset and their contribution to distinct microcircuit modules (Bikoff et al., 2016; Borowska et al., 2013; Goetz et al., 2015; Hayashi et al., 2018; Hilde et al., 2016; Tripodi et al., 2011). These data support a model wherein correct localization of spinal IN subsets is critical for proper formation of sensory-motor circuits, highlighting the importance of a strict regulation of short-distance neuronal migration in the developing spinal cord. However, genetic determinants that control spinal IN migration have only been sparsely identified (Blacklaws et al., 2015; Harris et al., 2019; Hilde et al., 2016; Kabayiza et al., 2017; Masgutova et al., 2019). Furthermore, non-cell autonomous mechanisms contribute to this control. Indeed, commissural axons have been shown to preconfigure ventral IN cell body position in the contra-lateral side of the spinal cord, acting either as border landmarks or as cellular guides for migration (Laumonnerie et al., 2015). This highlights a complex interplay between neuronal identity and neuronal positioning, jointly controlled by cell autonomous and non-cell autonomous mechanisms. Given that IN differentiation and IN distribution are affected

upon *Ntf3* deletion from MNs, separate contribution to one or the other process remains to be investigated. Transcriptomic characterization of alterations in pre-motor INs upon changes in Ntf3 production should address this question.

Ntf3 has been implicated in non-cell autonomous processes in other parts of the central nervous system. For example, in the developing cortex, Ntf3 produced by postmitotic neurons modulates the fate of progenitor cells during radial expansion (Parthasarathy et al., 2014). In the avian inner ear, it is required for proper development of the intrinsic properties of low-frequency neurons of the tonotopic axis in the cochlea (Takahashi and Sanchez, 2020), and could have a role in the postnatal inner ear activity, where it is transported by cochlear axons into the ventral cochlear nucleus (Feng et al., 2010). Furthermore, work in the chicken retina demonstrated that retinal ganglion cells can produce Ntf3 and transport it towards the optic tectum to control the survival and connectivity of superficial tectum cells (von Bartheld and Butowt, 2000). Importantly, spinal MNs have been shown to be necessary for survival and for peripheral or central projections of sensory neurons, and Ntf3 produced by the MNs partly contributes to survival and central projections of proprioceptive neurons, likely through transaxonal signaling (Usui et al., 2012). Therefore, the motor defects we observed in the MN-specific *Ntf3* mutants likely result from combined alterations of proprioceptive inputs and of the intrinsic organization and activity of spinal motor circuits. Nevertheless, our differentiation and distribution studies demonstrate a direct impact of Ntf3 produced in MNs on the development of spinal INs. Thus, Ntf3 contributes to regulate neuronal differentiation, survival, connectivity and activity in multiple neural structures.

At early stages of spinal cord development, *Ntf3* expression was restricted to MNs and its cognate receptor TrkC was detected in surrounding IN populations, consistent with previous reports (Buck et al., 2000; Henderson et al., 1993; Usui et al., 2012). OC factors moderate *Ntf3* expression levels in MNs, as previously observed for *Nkx6.2* (Toch et al., 2020) or for *Pou2f2* in ventral and in dorsal IN populations (Harris et al., 2019; Masgutova et al., 2019), suggesting that Ntf3 production in MNs must remain moderate to ensure proper development of pre-motor INs. Increased Ntf3 production has previously been shown to disrupt nervous system development or activity. *Ntf3* overexpression alters neuronal differentiation in the neocortex (Parthasarathy et al., 2014). In inner ear supporting cells or hair cells, it increased ribbon synapse density in postnatal cochlea and reduced auditory brainstem response thresholds at high frequencies (Wan et al., 2014). Increased production in the dorsal amygdala resulted in reduced anxious temperament and altered function of corresponding neural circuit (Fox et al., 2019). Thus, controlled levels of Ntf3 are critical for proper development and homeostasis of the nervous system.

Beside passive diffusion of signaling molecules in the extracellular space or transaxonal signaling as observed for Ntf3 (Usui et al., 2012), alternative mechanisms could additionally contribute to non-cell autonomous regulation of CNS development. Extracellular vesicles, which are also produced in the developing human spinal cord (Cau et al., 2022), have recently been shown to regulate the dorso-ventral differentiation and distribution of cortical INs. Extracellular vesicles produced in the ventral regions of the developing cortex contain secreted molecules, membrane proteins and ligand/receptor partners, and modulate fate and migration of ventrally-produced cortical INs (Pipicelli et al., 2023). Even more surprisingly, tunnelling nanotubes able to transfer molecules, endosomes and even mitochondria have recently been observed in living zebrafish embryos (Korenkova et al., 2025). Whether these transfer modalities are also in action in the mouse developing spinal cord remains to be assessed.

Materials and methods

Ethic statement and mouse lines

All experiments were performed strictly in accordance with the European directive 2010/63/UE. Mice were maintained in a conventional facility and fed in standard conditions (mice maintenance and mice breeding diets, Carfil Quality, Belgium), on a 14 h light/10 h dark cycle.

Food and water were available *ad libitum*. Experimental procedures on animals were approved by the animal ethics committees of the Université catholique de Louvain (Permit Numbers: 2013/UCL/MD/11; 2017/UCL/MD/008; 222801). The mutant strain mice were crossed and the morning the vaginal plug was detected was defined as embryonic day (e) 0.5. A minimum of three embryos (n≥3) of the same genotype was analyzed in each experiment. The embryos were harvested at embryonic days (e)10.5, e11.5, e12.5 or e14.5 depending on the mouse line. Constitutive $Oc1^{+/-}$;$Oc2^{+/-}$ mutants were crossed to obtain $Oc1^{-/-}$;$Oc2^{-/-}$ double-knockout embryos (Clotman et al., 2005). *Olig2|DTA* embryos, wherein the *Olig2* promoter drives expression of the diphteria toxin subunit A (DTA) in MN progenitors, have been obtained by crossing *Olig2-Cre* mice (Dessaud et al., 2007) with *Rosa26-flSTOP-DTA* mice (Ohnmacht et al., 2009). The $Oc1^{flox/flox}$; $Oc2^{flox/flox}$ mutant mice were crossed with Rosa26R- YFP/Olig2-Cre transgenic mice bearing heterozygous-null mutations for Oc1 and Oc2 genes (Rosa26-YFP;Olig2Cre;$Oc1^{+/-}$;$Oc2^{+/-}$) to obtain conditional double inactivation of Oc1 and Oc2 in MNs (Toch et al., 2020). The combined inactivation of Oc1 and Oc2 in MNs completely abolishes the expression of *Oc3* in the developing spinal cord (Kabayiza et al., 2017; Roy et al., 2012). $Ntf3^{+/flox}$ mice (B6.129 S4-Ntf3tm2Jae/J, The Jackson Laboratory) were crossed first with PGK-Cre mice to obtain a transgenic line with a constitutive heterozygous inactivation of *Ntf3* ($Ntf3^{+/-}$), then with the Olig2-Cre mice (Olig2Cre;$Ntf3^{+/-}$). The $Ntf3^{flox/flox}$ mutant mice were crossed with the Olig2Cre;$Ntf3^{+/-}$ mice to obtain a conditional inactivation of *Ntf3* in MNs. PCR genotyping protocols and primers are available upon request.

In situ hybridization and immunofluorescence

For *in situ* hybridization (ISH), collected embryos were fixed in ice-cold 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) overnight at 4 °C, washed thrice in PBS for 10 minutes, incubated in PBS/30% sucrose solution overnight at 4 °C, and embedded and frozen in PBS/15% sucrose/7.5% gelatin. Sixteen micrometer sections were prepared, and ISH was performed as previously described (Beguin et al., 2013; Francius et al., 2016; Pelosi et al., 2014) with DIG-conjugated Ntf3 antisense RNA probes (primer pair: 5'-GATCCAGGCGGATATCTTGA-3'and 5'-CGGACA TAGGTTTGCGAAGT-3). Control or Ntf3 conditional mutant sections were placed adjacent on the same histology slides to minimize inter-slide variations of ISH signals.

For immunofluorescence, collected embryos were immersion-fixed in 4% PFA/PBS for 15, 25 or 35 minutes at 4°C according to their embryonic stage, and processed as for ISH. Immunolabeling was performed on 14 µm serial cryosections as previously described (Debrulle et al., 2020; Francius et al., 2013). Primary antibodies against the following proteins were used: sheep anti-Chx10 (Exalpha Biologicals #X1179P) at 1:200 or guinea pig anti-Chx10 (Peng et al., 2007) at 1:3000, rabbit anti-cleaved Caspase-3 (Asp175) antibody (Cell Signaling #9664) at 1:1000, mouse anti-Evx1 (DSHB #99.1-3A2) at 1:2000 or rabbit anti-Evx1 (Moran- Rivard et al., 2001) at 1:300, rabbit anti-Foxd3 at 1:5000 or guinea pig anti-Foxd3 (Muller et al., 2005) at 1:5000, goat anti-Foxp2 (Abcam #ab1307) at 1:2000, guinea pig anti-Gata2 (Peng et al., 2007) at 1:3000, rat anti-Gata3 (Absea Biotechnology #111214D02) at 1:15 or rabbit anti-Gata3 (Cell signaling #5852) at 1:200, guinea pig anti-MafA (Gierl et al., 2006) at 1:500, goat anti-Isl1 (R&D #AF1837) at 1:1000, rabbit anti-MafA (Novus Biological #NB400-137) at 1:500, mouse anti-MNR2 (DSHB #81.5C10) at 1:1000, Mouse anti-Nkx2.2 (DSHB #74.5A5) at 1:20, rabbit anti-Otp at 1:1600 (ThermoFisher #PA5-89060), rabbit anti-Prdm8 at 1 :200 or mouse anti-Prdm8 at 1:200(Komai et al., 2009), goat anti-Sox1 (Santa Cruz #sc-17318) at 1:500 or rabbit anti-Sox1 (Panayi et al., 2010) at 1:400 and goat anti-TrkC (R&D Systems #AF1404) at 1:500. The secondary antibodies used are donkey anti-chicken/Alexafluor 488, donkey anti-goat/AlexaFluor 594, donkey anti-goat/AlexaFluor 488, donkey anti-goat/AlexaFluor 647, donkey anti-mouse/AlexaFluor 594, donkey anti-mouse/AlexaFluor 488, donkey anti-rabbit/AlexaFluor 594, donkey anti-rabbit/AlexaFluor 488, donkey anti-rabbit/AlexaFluor 647, donkey anti-rat/AlexaFluor 594, donkey anti-rat/AlexaFluor 488, donkey anti-guinea pig/Alexa fluor 488 purchased from ThermoFisher Scientific or Jackson Laboratories and used at 1:500 to 1:2000 dilutions.

Immunofluorescence and ISH images from cryosections were acquired on an EVOS FL Auto Imaging System (ThermoFisher Scientific), a Zeiss AxioSkop2 or Zeiss AXIO Observer Z1 epifluorescence microscopes. The images were processed using Adobe Photoshop CS5 or ImageJ (Fiji) softwares to match brightness and contrast. The rostrocaudal levels of the spinal cord were precisely aligned using the distribution of Foxp1 in the Lateral Motor Columns (LMCs) at brachial or lumbar levels of the spinal cord (Rousso et al., 2008; Roy et al., 2012).

In ovo electroporation

The *in ovo* electroporations of chicken embryonic spinal cord were performed at stage HH12 (around forty-eight hours of development), and embryos were collected 24, 48 or 72 h after electroporation. The coding sequence of *Ntf3* (primer pair: 5'-CGGATGCCATGGTTACTTCT-3' and 5'-TGCCAATTCATGTTCTTCCA-3') was cloned into the vector pHb9-MCS-IRES-EGFP to increase the expression of the gene in the MNs. pHb9-Ntf3-IRES-GFP vector (2 µg/µl) was electroporated using the EGFP signal to visualize cells expressing *Ntf3*. However, given the transient expression of such constructs after electroporation, maximal GFP production could only be visualized 24h after electroporation (data not shown). The collected embryos were fixed in ice-cold PBS/4% PFA for 15, 20 or 25 minutes, depending on their stage, and processed as described above for ISH or immunofluorescence. The MNs and ventral IN populations were analyzed using 5-10 sections per embryo, the contralateral side of the spinal cord being considered as a perfect stage- and experimental condition-matching control. The labelled cells were counted on both sides of the spinal cord, with the counting tool of Image J (Fiji).

FACS, RNA purification and RNA-sequencing

Spinal cords from e10.5 control or Rosa26-YFP;Olig2Cre;$Oc1^{\Delta/-}$;$Oc2^{\Delta/-}$ embryos were harvested and dissociated using a neural tissue dissociation kit (MACS; Miltenyi Biotec #130-092-628), dissociated cells were sorted by FACS (BD FACSAria III) to collect YFP-positive cells. The sorted cells were collected in TRIzol reagent, and RNA was purifed with the Rneasy micro kit (QIAGEN #74004). RNA concentration and quality were assessed using a Bioanalyzer (Agilent) and submitted to Genewiz to prepare an ultra-low input RNA-seq library before sequencing with an Illumina HiSeq. Preliminary data were analyzed by Genewiz using the standard RNA-seq data analysis package. RNAseq data have been deposited in the GEO repository (accession number: GSE141949) (Toch et al., 2020).

Motor behavior tests in adult mice

Behavior tests were performed on male (n=8) or female (n=8) mice, comparing control Olig2Cre;$Ntf3^{+/-}$ mice with Olig2Cre;$Ntf3^{\Delta/-}$ conditional mutant mice which lack Ntf3 in MNs, starting at two months of age. The rotarod and the balance beam tests were performed as previously described (Audouard et al., 2012; Renaux et al., 2024).

Statistical analyses

For each of the embryos analyzed (n≥3), neuron quantifications were performed using the count analysis tool of ImageJ on sections of the spinal cord at brachial, thoracic or lumbar levels, except for chicken embryos that are too early to delineate different levels in the spinal cord. Graphs were generated using R. The statistical tests applied to compare the cell count comparing two groups (controls versus constitutive or conditional mutants) was performed in R [R Core Team 2024] (version 4.4.2) either with a mixed model (using the R packages lme4 (Bates et al., 2015) version 1.1-37 and lmerTest (Kuznetsova et al., 2017) version 3.1- 3) involving a random embryo effect if the random effect was significant or a linear regression if it was deemed non-significant. A correction for multiple testing with the False Discovery Rate procedure was applied to the obtained p-values.

The spatial distribution of the motor neurons and ventral IN populations was analyzed using a gradient map. In a transverse section of the spinal cord, the height (H) was defined as the distance from the ventral limit of the central canal to the most dorsal edge of the spinal cord, and the width (W) was the distance from the central canal to the lateral edge. For each population, several

sections were analyzed at brachial, thoracic and lumbar levels. The distance (dIN) and angle (αIN) were measured from the ventral limit of the central canal to the cell soma using the ruler analysis tool in Image J. The dorso-ventral (DV) and medio-lateral (ML) positions of the cells were expressed as percentages of spinal cord height and hemicord width respectively: DV position and ML position were defined as (dIN ∗ sin αIN)/H and (dIN ∗ cos αIN)/W, respectively, and ML versus DV values were plotted using R with a 2D kernel density (Harris et al., 2019; Kabayiza et al., 2017). For each section and level, a non parametric multivariate ANOVA-type statistic was performed (R package npmv, version 2.4 (Burchett et al., 2017)) to assess if the locations of DV and ML in the two conditions are different.

For the balance beam and rotarod tests, differences between the two experimental groups were evaluated with the JMP software using either a Wilcoxon-Mann-Whitney's non parametrical test, a Welch's *t*-test or a Student's *t*-test, depending on the normality and the homoscedasticity of the data. In all statistical analyses, a value of $p < 0.05$ was defined as significant.

The complete statistical analysis is available here: *https://github.com/ManonMartin/Non-cell_autonomous_regulation_of_spinal_motor_circuit_development_Angla-Navarro-Dominguez-Bajo*

Data availability

RNA-sequencing data were deposited in the GEO repository, accession number: GSE141949 The whole statistical workflow used in the study is available on the GitHub platform

Supplementary figures

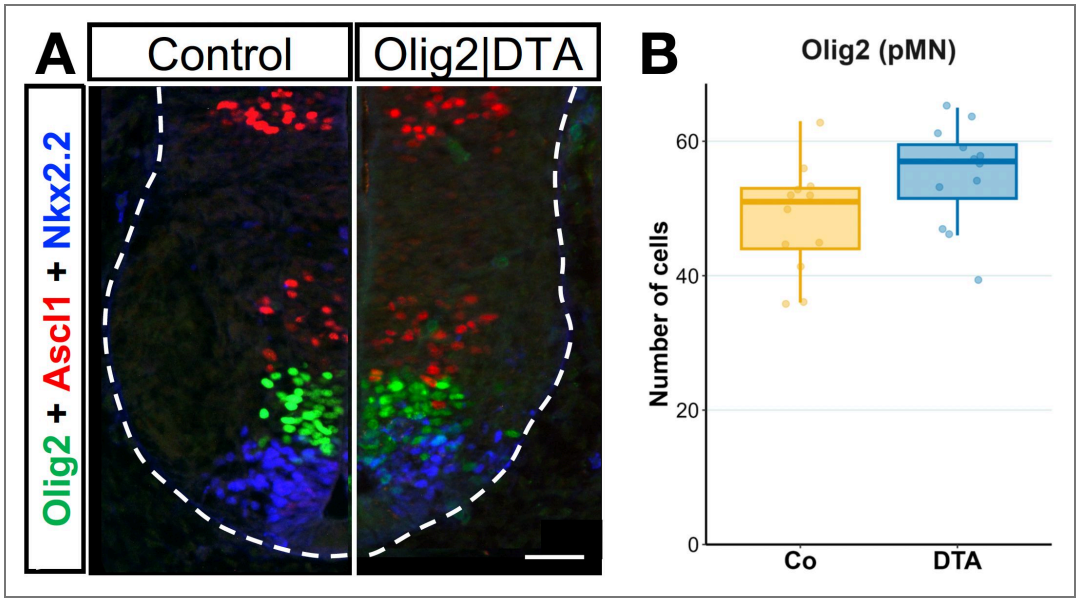


Figure S1. The MN progenitor domain is not altered in *Olig2|DTA* embryos. At e10.5, the number of MN progenitors characterized by the presence of Olig2 (green) in the absence of Ascl1 (V2 progenitors, red) or Nkx2.2 (V3 progenitors, blue) is similar to control embryos. Scale bar=100μm.

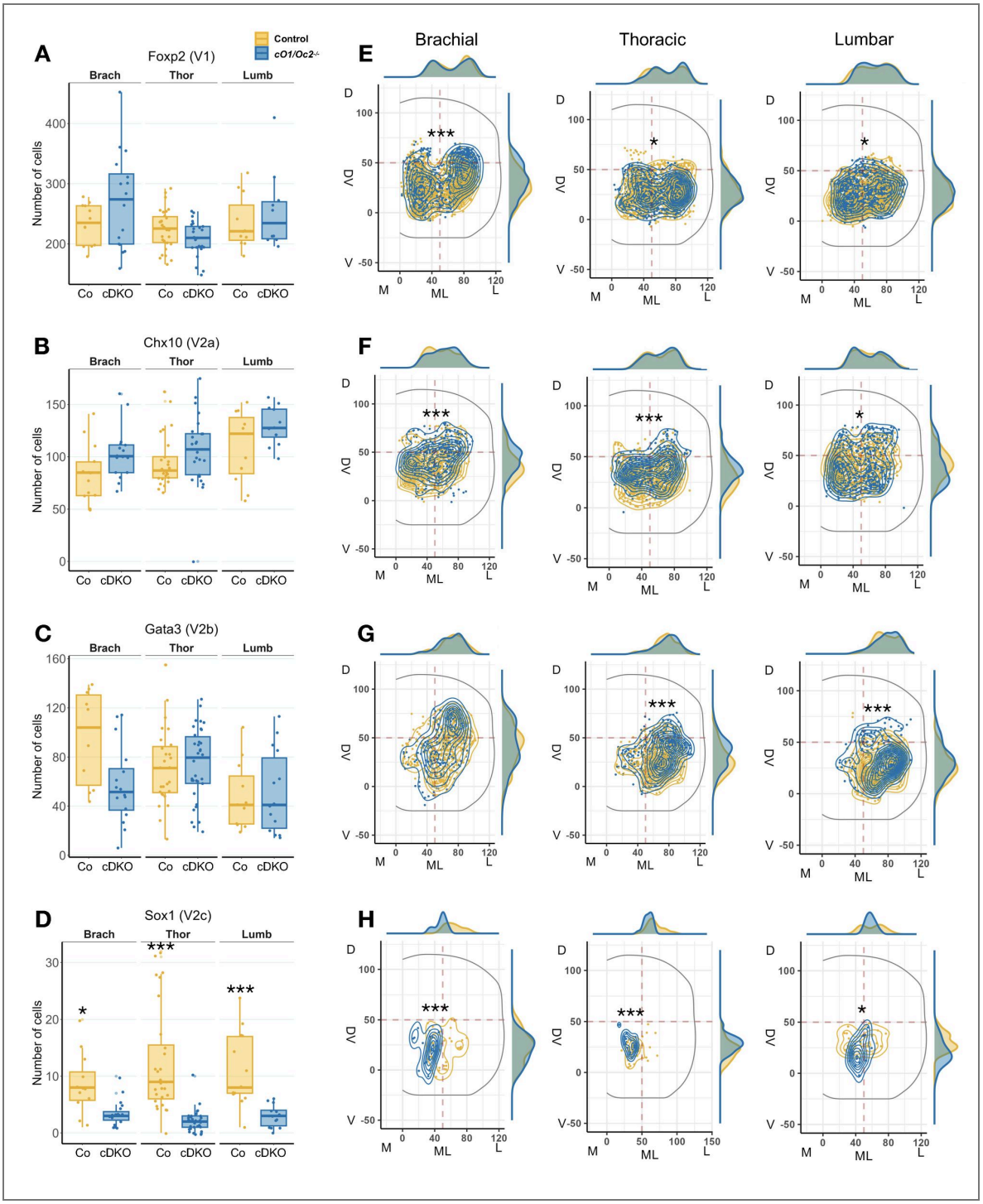


Figure S2. Spinal interneuron number and positioning is altered when Onecut factors are absent from the spinal motor neurons.

(A-H) In MN conditional *Oc1/Oc2* double-mutant embryos (cDKO) at e14.5, the number of V2c INs identified by the presence of Sox1 outside of the ventricular zone was significantly reduced at all levels of the spinal cord. Furthermore, the positioning of the V1 (Foxp2), V2a (Chx10), V2b (Gata3) and V2c INs on the transversal plane of the spinal cord was altered. ML: medio-lateral axis; DV: dorso-ventral axis. n=3; *=adjusted p value < 0.05; **=adjusted p value < 0.01; ***=adjusted p value < 0.001

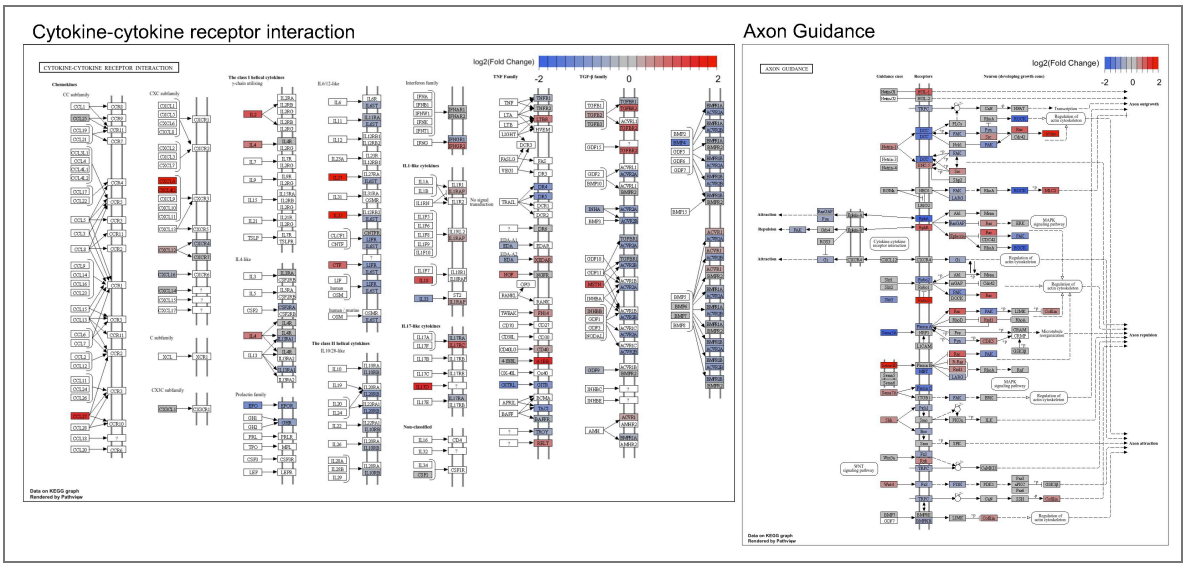


Figure S3. Pathway analysis of down- or up-regulated genes in spinal MN deficient in Onecut factors.

Genes showing altered expression levels are enriched in the “cytokine-cytokine receptor interaction” and in the “Axon guidance” pathways. Down-regulated genes are in blue and up-regulated genes in red (see GEO repository, accession number GSE141949 for more details).

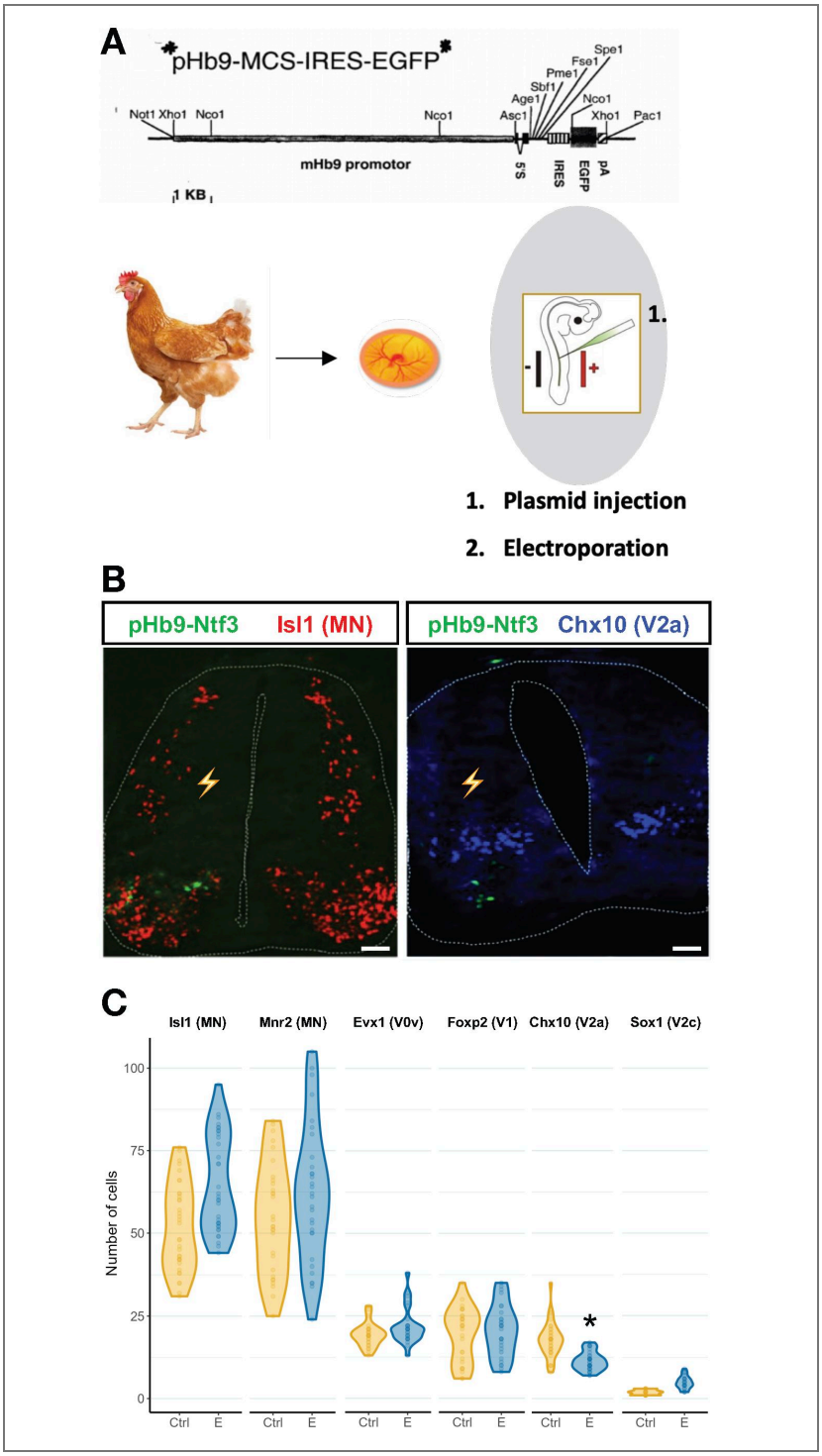


Figure S4. Ntf3 overexpression after chicken embryonic spinal cord electroporation mildly impacts spinal interneuron development.

(A) The pHb9-Ntf3-IRES-EGFP expression vector was electroporated in the chicken embryonic spinal cord. **(B)** Immunofluorescence labelings for Isl1 to identify MNs (given its transient expression, maximal GFP production could only be visualized 24h after electroporation) and Chx10 to visualize V2a INs 48h after electroporation. **(C)** Quantification of IN populations in control (Ctrl, contralateral sides) or electroporated (E) sides of the spinal cord. The number of V2a INs was mildly decreased after *Ntf3* overexpression, while other IN populations were not affected. Scale bars=100µm. n=3; *=adjusted p value < 0.05

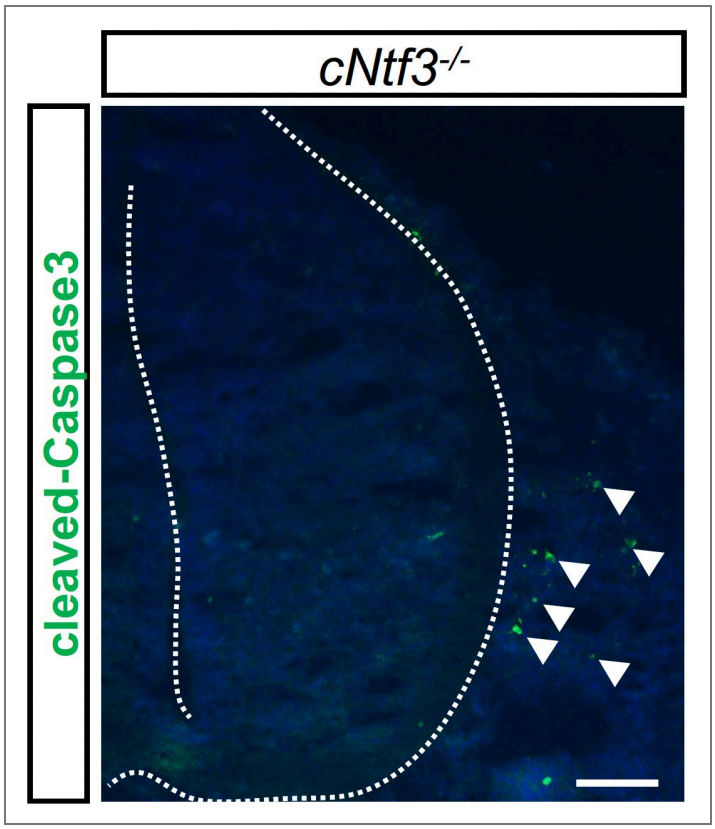


Figure S5. Ntf3 inactivation does not increase spinal cord cell death.

Conditional inactivation of *Ntf3* in MNs does not induce cell death in the spinal cord at e12.5, as evidenced by the absence of cleaved-Caspase3. Cell death of sensory neurons in the dorsal root ganglia (arrowheads) served as a positive labeling control.

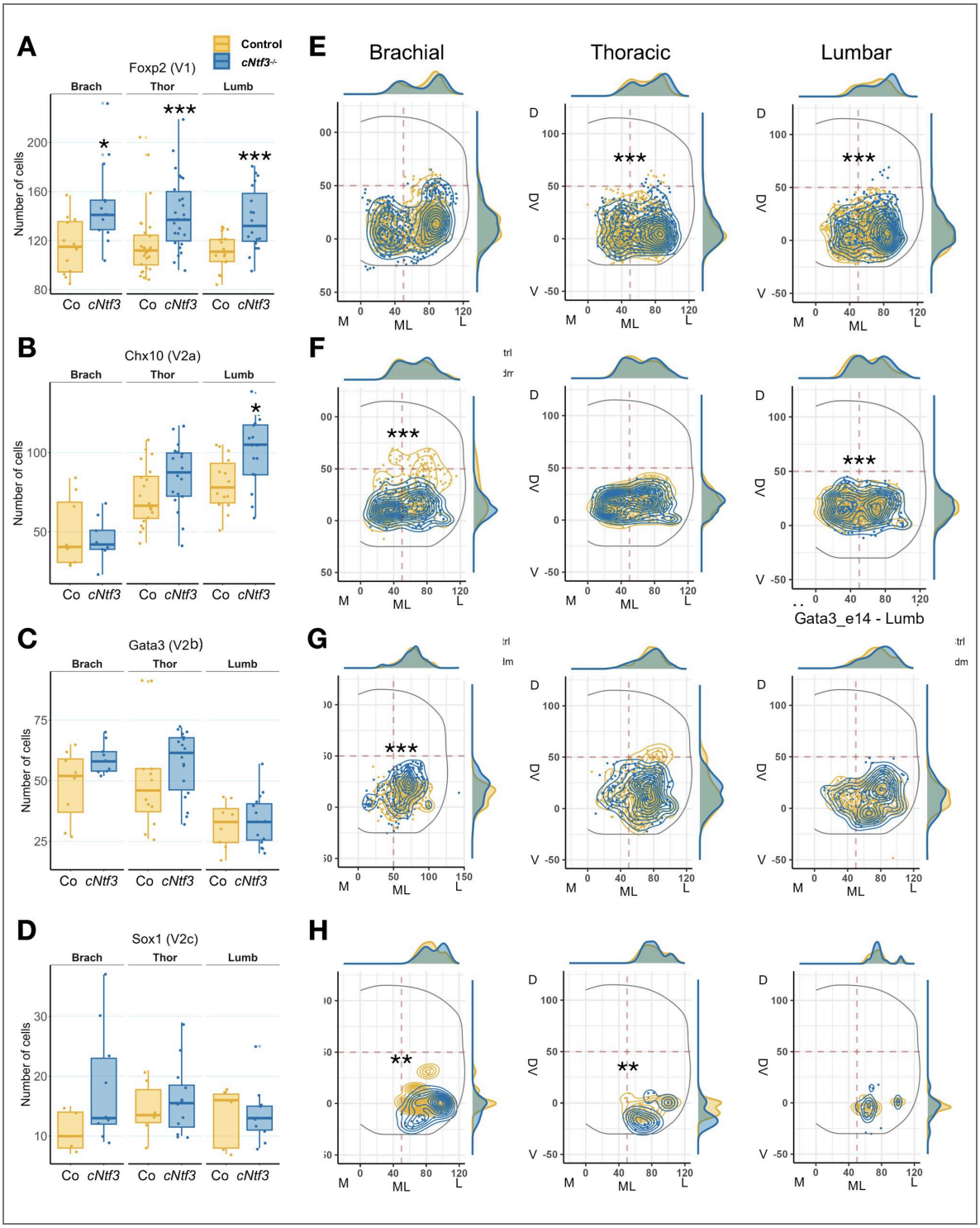


Figure S6. Spinal interneuron number and positioning is altered when Ntf3 is inactivated in the spinal motor neurons.

(A-H) In MN conditional *Ntf3* mutant embryos (*cNtf3*) at e14.5, the number of V1 (Foxp2) INs was significantly increased at all levels of the spinal cord, while V2a (Chx10) only expanded at lumbar level and V2b (Gata3) or V2c (Sox1, white arrows) were not altered. Furthermore, the positioning of all these IN populations on the transversal plane of the spinal cord was altered at all levels of the spinal cord. ML: medio-lateral axis; DV: dorso-ventral axis. n=3; *=adjusted p value < 0.05; **=adjusted p value < 0.01; ***=adjusted p value < 0.001

Acknowledgements

We thank previous members of the NEDI lab and members of the AMCB group for material, technical support and discussions, Coralie Piget at the “Animalerie Carnoy” (ANCA) facility for expert assistance in mouse colony care and management, and Catherine Rasse for expert input on quantifications and statistical analyses. We are indebted to Pr. Yoichi Shinkai, Dr. Stavros Malas, Dr. Martyn Goulding, Dr. Kamal Sharma, Dr. Sam Pfaff, Dr. Thomas Müller, Dr. Frank Grosveld, Dr. Yoichi Shinkai, Dr. Sarah E. Ross and Dr. Stavros Malas for antibodies and reagents, and to Dr. Andrea B. Huber for the generous gift of *Olig2|DTA* embryo sections. The anti-Evx1, -MNR2 and -Nkx2.2 antibodies were obtained from the Developmental Studies Hybridoma Bank, created by the NICHD of the NIH and maintained at The University of Iowa, Department of Biology, Iowa City, IA 52242. Research in the FC group is supported by grants from the « Fonds spéciaux de recherche » (FSR) of the Université catholique de Louvain, by the “Actions de Recherche Concertées (ARC)” #17/22-079 of the “Direction générale de l’Enseignement non obligatoire et de la Recherche scientifique – Direction de la Recherche scientifique – Communauté française de Belgique” and granted by the “Académie universitaire ‘Louvain’”, by a “Projet de recherche (PDR)” #T.0117.13 and an “Equipement (EQP)” funding #U.N027.14 of the Fonds de la Recherche Scientifique (F.R.S.-FNRS) and by the Association Belge contre les Maladies neuro-Musculaires (ABMM). Work in XM lab is supported by grants from the National Eye Institute of the National Institutes of Health (R01EY020545 and R01EY029705). AAN held a PhD grant from the F.R.S.-FNRS, Belgium; MT held a specialization grant from the FRIA (F.R.S.-FNRS); JZ holds a PhD grant from the China Scholarship Council (CSC, No. 202208440068) and a UCLouvain-CSC Ph.D. co-funding fellowship from UCLouvain; MF holds a PhD grant from the Fonds Spéciaux de Recherche (FSR) from UClouvain; ADB and MHF held a postdoctoral researcher grant from the F.R.S.-FNRS. FC is a Research Director of the F.R.S.-FNRS.

Additional information

Author contribution

AAN: Conceptualization, Investigation, Data analysis, Data interpretation, Writing - original draft

ADB: Conceptualization, Investigation, Data analysis, Data interpretation, Writing – original draft

MT: Conceptualization, Investigation, Data analysis, Data interpretation

CF: Conceptualization, Investigation, Data analysis, Data interpretation

MHF: Conceptualization, Investigation, Data analysis, Data interpretation

JZ: Investigation, Data analysis

MF: Investigation, Data analysis

OS: Conceptualization, Investigation, Data analysis

MM: Data analysis

XM: Provided experimental model

RR: Data interpretation

FG: Data interpretation

FC: Conceptualization, Supervision, Funding acquisition, Data interpretation, Writing – original draft

Funding

Funder	Grant reference number	Author
AUL | Université Catholique de Louvain (UCL)	FSR 2020-2021	Frederic Clotman
AUL | Université Catholique de Louvain (UCL)	ARC #17/22-079	Frederic Clotman

Fonds De La Recherche Scientifique - FNRS (FNRS)	PDR #T.0117.13	Frederic Clotman
Fonds De La Recherche Scientifique - FNRS (FNRS)	EQP #U.N027.14	Frederic Clotman
Association Belge contre les Maladies Neuro-Musculaires (ABMM)	2023 and 2025 grants	Frederic Clotman
HHS | NIH | National Eye Institute (NEI)	R01EY020545	Xiuqian Mu
HHS | NIH | National Eye Institute (NEI)	R01EY029705	Xiuqian Mu

Author ORCID iDs

Xiuqian Mu: https://orcid.org/0000-0002-8003-7529
Frédéric Clotman: https://orcid.org/0000-0002-0497-2195

References

Allan DW, Thor S (2015) Transcriptional selectors, masters, and combinatorial codes: regulatory principles of neural subtype specification. *Wiley Interdiscip Rev Dev Biol* **4**:505-528 https://doi.org/10.1002/wdev.191 | PubMed

Audouard E, Schakman O, Rene F, Huettl RE, Huber AB, Loeffler JP, Gailly P, Clotman F (2012) The Onecut transcription factor HNF-6 regulates in motor neurons the formation of the neuromuscular junctions. *PLoS One* **7**:e50509 https://doi.org/10.1371/journal.pone.0050509 | PubMed

Barber M, Arai Y, Morishita Y, Vigier L, Causeret F, Borello U, Ledonne F, Coppola E, Contremoulins V, Pfrieger FW, *et al.* (2015) Migration Speed of Cajal-Retzius Cells Modulated by Vesicular Trafficking Controls the Size of Higher-Order Cortical Areas. *Curr Biol* **25**:2466-2478 https://doi.org/10.1016/j.cub.2015.08.028 | PubMed

Bechade C, Mallecourt C, Sedel F, Vyas S, Triller A (2002) Motoneuron-derived neurotrophin-3 is a survival factor for PAX2-expressing spinal interneurons. *J Neurosci* 8779-8784 https://doi.org/10.1523/jneurosci.22-20-08779.2002 | PubMed

Beguin S, Crepel V, Aniksztejn L, Becq H, Pelosi B, Pallesi-Pocachard E, Bouamrane L, Pasqualetti M, Kitamura K, Cardoso C, *et al.* (2013) An epilepsy-related ARX polyalanine expansion modifies glutamatergic neurons excitability and morphology without affecting GABAergic neurons development. *Cereb Cortex* **23**:1484-1494 https://doi.org/10.1093/cercor/bhs138 | PubMed

Bernard C, Vincent C, Testa D, Bertini E, Ribot J, Di Nardo AA, Volovitch M, Prochiantz A (2016) A Mouse Model for Conditional Secretion of Specific Single-Chain Antibodies Provides Genetic Evidence for Regulation of Cortical Plasticity by a Non-cell Autonomous Homeoprotein Transcription Factor. *PLoS Genet* **12**:e1006035 https://doi.org/10.1371/journal.pgen.1006035 | PubMed

Bikoff JB, Gabitto MI, Rivard AF, Drobac E, Machado TA, Miri A, Brenner-Morton S, Famojure E, Diaz C, Alvarez FJ, *et al.* (2016) Spinal Inhibitory Interneuron Diversity Delineates Variant Motor Microcircuits. *Cell* **165**:207-219 https://doi.org/10.1016/j.cell.2016.01.027 | PubMed

Blacklaws J, Deska-Gauthier D, Jones CT, Petracca YL, Liu M, Zhang H, Fawcett JP, Glover JC, Lanuza GM, Zhang Y (2015) Sim1 is required for the migration and axonal projections of V3 interneurons in the developing mouse spinal cord. *Dev Neurobiol* **75**:1003-1017 https://doi.org/10.1002/dneu.22266 | PubMed

Borowska J, Jones CT, Zhang H, Blacklaws J, Goulding M, Zhang Y (2013) Functional subpopulations of V3 interneurons in the mature mouse spinal cord. *J Neurosci* **33**:18553-18565 https://doi.org/10.1523/jneurosci.2005-13.2013 | PubMed

Buck CR, Seburn KL, Cope TC (2000) Neurotrophin expression by spinal motoneurons in adult and developing rats. *J Comp Neurol* **416**:309-318 https://doi.org/10.1002/(sici)1096-9861(20000117)416:3<309::aid-cne3>3.0.co;2-u | PubMed

Burchett WW, Ellis AR, Harrar SW, Bathke AC (2017) Nonparametric Inference for Multivariate Data: The R Package npmv. *Journal of Statistical Software* **76**:1-18 https://doi.org/10.18637/jss.v076.i04

Cau F, Fanni D, Manchia M, Gerosa C, Piras M, Murru R, Paribello P, Congiu T, Coni P, Pichiri G, *et al.* (2022) Expression of L1 Cell Adhesion Molecule (L1CAM) in extracellular vesicles in the human spinal cord during development. *Eur Rev Med Pharmacol Sci* **26**:6273-6282 https://doi.org/10.26355/eurrev_202209_29651 | PubMed

Causeret F, Ensini M, Teissier A, Kessaris N, Richardson WD, Lucas de Couville T, Pierani A (2011) Dbx1-expressing cells are necessary for the survival of the mammalian anterior neural and craniofacial structures. *PLoS One* **6**:e19367 https://doi.org/10.1371/journal.pone.0019367 | PubMed

Chen JA, Huang YP, Mazzoni EO, Tan GC, Zavadil J, Wichterle H (2011) Mir-17-3p controls spinal neural progenitor patterning by regulating Olig2/Irx3 cross-repressive loop. *Neuron* **69**:721-735 https://doi.org/10.1016/j.neuron.2011.01.014 | PubMed

Choi BR, Cave C, Na CH, Sockanathan S (2020) GDE2-Dependent Activation of Canonical Wnt Signaling in Neurons Regulates Oligodendrocyte Maturation. *Cell Rep* **31**:107540 https://doi.org/10.1016/j.celrep.2020.107540 | PubMed

Clotman F, Jacquemin P, Plumb-Rudewiez N, Pierreux CE, Van der Smissen P, Dietz HC, Courtoy PJ, Rousseau GG, Lemaigre FP (2005) Control of liver cell fate decision by a gradient of TGF beta signaling modulated by Onecut transcription factors. *Genes Dev* **19**:1849-1854 https://doi.org/10.1101/gad.340305 | PubMed

Co M, Hickey SL, Kulkarni A, Harper M, Konopka G (2020) Cortical Foxp2 Supports Behavioral Flexibility and Developmental Dopamine D1 Receptor Expression. *Cereb Cortex* **30**:1855-1870 https://doi.org/10.1093/cercor/bhz209 | PubMed

Cote MP, Murray LM, Knikou M (2018) Spinal Control of Locomotion: Individual Neurons, Their Circuits and Functions. *Front Physiol* **9**:784 https://doi.org/10.3389/fphys.2018.00784 | PubMed

Cunningham JG, Scripter JD, Nti SA, Tucker ES (2022) Early construction of the thalamocortical axon pathway requires c-Jun N-terminal kinase signaling within the ventral forebrain. *Dev Dyn* **251**:459-480 https://doi.org/10.1002/dvdy.416 | PubMed

Czesnick H, Lenhard M (2015) Size control in plants--lessons from leaves and flowers. *Cold Spring Harb Perspect Biol* **7**:a019190 https://doi.org/10.1101/cshperspect.a019190 | PubMed

D'Arcy BR, Lennox AL, Manso Musso C, Bracher A, Escobar-Tomlienovich C, Perez-Sanchez S, Silver DL (2023) Non-muscle myosins control radial glial basal endfeet to mediate interneuron organization. *PLoS Biol* **21**:e3001926 https://doi.org/10.1371/journal.pbio.3001926 | PubMed

Debrulle S, Baudouin C, Hidalgo-Figueroa M, Pelosi B, Francius C, Rucchin V, Ronellenfitch K, Chow RL, Tissir F, Lee SK, *et al.* (2020) Vsx1 and Chx10 paralogs sequentially secure V2 interneuron identity during spinal cord development. *Cell Mol Life Sci* **77**:4117-4131 https://doi.org/10.1007/s00018-019-03408-7 | PubMed

Dessaud E, Yang LL, Hill K, Cox B, Ulloa F, Ribeiro A, Mynett A, Novitch BG, Briscoe J (2007) Interpretation of the sonic hedgehog morphogen gradient by a temporal adaptation mechanism. *Nature* **450**:717-720 https://doi.org/10.1038/nature06347 | PubMed

Di Nardo AA, Joliot A, Prochiantz A (2020) Homeoprotein transduction in neurodevelopment and physiopathology. *Sci Adv* **6** https://doi.org/10.1126/sciadv.abc6374 | PubMed

Dutton R, Yamada T, Turnley A, Bartlett PF, Murphy M (1999) Sonic hedgehog promotes neuronal differentiation of murine spinal cord precursors and collaborates with neurotrophin 3 to induce Islet-1. *J Neurosci* **19**:2601-2608 https://doi.org/10.1523/jneurosci.19-07-02601.1999 | PubMed

El Wakil A, Francius C, Wolff A, Pleau-Varet J, Nardelli J (2006) The GATA2 transcription factor negatively regulates the proliferation of neuronal progenitors. *Development* **133**:2155-2165 https://doi.org/10.1242/dev.02377 | PubMed

ElShamy WM, Ernfors P (1996a) A local action of neurotrophin-3 prevents the death of proliferating sensory neuron precursor cells. *Neuron* **16**:963-972 https://doi.org/10.1016/s0896-6273(00)80119-1 | PubMed

elshamy WM, Ernfors P (1996b) Requirement of neurotrophin-3 for the survival of proliferating trigeminal ganglion progenitor cells. *Development* **122**:2405-2414 https://doi.org/10.1242/dev.122.8.2405 | PubMed

Feng J, Bendiske J, Morest DK (2010) Postnatal development of NT3 and TrkC in mouse ventral cochlear nucleus. *J Neurosci Res* **88**:86-94 https://doi.org/10.1002/jnr.22179 | PubMed

Fox AS, Souaiaia T, Oler JA, Kovner R, Kim JMH, Nguyen J, French DA, Riedel MK, Fekete EM, Rabska MR, *et al.* (2019) Dorsal Amygdala Neurotrophin-3 Decreases Anxious Temperament in Primates. *Biol Psychiatry* **86**:881-889 https://doi.org/10.1016/j.biopsych.2019.06.022 | PubMed

Francius C, Clotman F (2010) Dynamic expression of the Onecut transcription factors HNF-6, OC-2 and OC-3 during spinal motor neuron development. *Neuroscience* **165**:116-129 https://doi.org/10.1016/j.neuroscience.2009.09.076 | PubMed

Francius C, Clotman F (2014) Generating spinal motor neuron diversity: a long quest for neuronal identity. *Cell Mol Life Sci* **71**:813-829 https://doi.org/10.1007/s00018-013-1398-x | PubMed

Francius C, Harris A, Rucchin V, Hendricks TJ, Stam FJ, Barber M, Kurek D, Grosveld FG, Pierani A, Goulding M, *et al.* (2013) Identification of multiple subsets of ventral interneurons and differential distribution along the rostrocaudal axis of the developing spinal cord. *PLoS One* **8**:e70325 https://doi.org/10.1371/journal.pone.0070325 | PubMed

Francius C, Hidalgo-Figueroa M, Debrulle S, Pelosi B, Rucchin V, Ronellenfitch K, Panayiotou E, Makrides N, Misra K, Harris A, *et al.* (2016) Vsx1 Transiently Defines an Early Intermediate V2 Interneuron Precursor Compartment in the Mouse Developing Spinal Cord. *Front Mol Neurosci* **9**:145 https://doi.org/10.3389/fnmol.2016.00145 | PubMed

Gibel-Russo R, Benacom D, Di Nardo AA (2022) Non-Cell-Autonomous Factors Implicated in Parvalbumin Interneuron Maturation and Critical Periods. *Front Neural Circuits* **16**:875873 https://doi.org/10.3389/fncir.2022.875873 | PubMed

Gierl MS, Karoulias N, Wende H, Strehle M, Birchmeier C (2006) The zinc-finger factor Insm1 (IA-1) is essential for the development of pancreatic beta cells and intestinal endocrine cells. *Genes Dev* **20**:2465-2478 https://doi.org/10.1101/gad.381806 | PubMed

Gil-Sanz C, Franco SJ, Martinez-Garay I, Espinosa A, Harkins-Perry S, Muller U (2013) Cajal-Retzius cells instruct neuronal migration by coincidence signaling between secreted and contact-dependent guidance cues. *Neuron* **79**:461-477 https://doi.org/10.1016/j.neuron.2013.06.040 | PubMed

Goetz C, Pivetta C, Arber S (2015) Distinct limb and trunk premotor circuits establish laterality in the spinal cord. *Neuron* **85**:131-144 https://doi.org/10.1016/j.neuron.2014.11.024 | PubMed

Grieshammer U, Lewandoski M, Prevette D, Oppenheim RW, Martin GR (1998) Muscle-specific cell ablation conditional upon Cre-mediated DNA recombination in transgenic mice leads to massive spinal and cranial motoneuron loss. *Dev Biol* **197**:234-247 https://doi.org/10.1006/dbio.1997.8859 | PubMed

Guemez-Gamboa A, Xu L, Meng D, Spitzer NC (2014) Non-cell-autonomous mechanism of activity-dependent neurotransmitter switching. *Neuron* **82**:1004-1016 https://doi.org/10.1016/j.neuron.2014.04.029 | PubMed

Harris A, Masgutova G, Collin A, Toch M, Hidalgo-Figueroa M, Jacob B, Corcoran LM, Francius C, Clotman F (2019) Onecut factors and Pou2f2 regulate the distribution of V2 interneurons in the mouse developing spinal cord. *Front Cell Neurosci* **13** https://doi.org/10.3389/fncel.2019.00184 | PubMed

Hayashi M, Hinckley CA, Driscoll SP, Moore NJ, Levine AJ, Hilde KL, Sharma K, Pfaff SL (2018) Graded Arrays of Spinal and Supraspinal V2a Interneuron Subtypes Underlie Forelimb and Hindlimb Motor Control. *Neuron* **97**:869-884. https://doi.org/10.1016/j.neuron.2018.01.023 | PubMed

Henderson CE, Camu W, Mettling C, Gouin A, Poulsen K, Karihaloo M, Rullamas J, Evans T, McMahon SB, Armanini MP, *et al.* (1993) Neurotrophins promote motor neuron survival and are present in embryonic limb bud. *Nature* **363**:266-270 https://doi.org/10.1038/363266a0 | PubMed

Hilde KL, Levine AJ, Hinckley CA, Hayashi M, Montgomery JM, Gullo M, Driscoll SP, Grosschedl R, Kohwi Y, Kohwi-Shigematsu T, *et al.* (2016) Satb2 Is Required for the Development of a Spinal Exteroceptive Microcircuit that Modulates Limb Position. *Neuron* **91**:763-776 https://doi.org/10.1016/j.neuron.2016.07.014 | PubMed

Itoh Y, Sahni V, Shnider SJ, McKee H, Macklis JD (2023) Inter-axonal molecular crosstalk via Lumican proteoglycan sculpts murine cervical corticospinal innervation by distinct subpopulations. *Cell Rep* **42**:112182 https://doi.org/10.1016/j.celrep.2023.112182 | PubMed

Jossin Y (2020) Reelin Functions, Mechanisms of Action and Signaling Pathways During Brain Development and Maturation. *Biomolecules* **10** https://doi.org/10.3390/biom10060964 | PubMed

Kabayiza KU, Masgutova G, Harris A, Rucchin V, Jacob B, Clotman F (2017) The Onecut Transcription Factors Regulate Differentiation and Distribution of Dorsal Interneurons during Spinal Cord Development. *Front Mol Neurosci* **10**:157 https://doi.org/10.3389/fnmol.2017.00157 | PubMed

Kablar B, Rudnicki MA (1999) Development in the absence of skeletal muscle results in the sequential ablation of motor neurons from the spinal cord to the brain. *Dev Biol* **208**:93-109 https://doi.org/10.1006/dbio.1998.9184 | PubMed

Kaddour H, Coppola E, Di Nardo AA, Le Poupon C, Mailly P, Wizenmann A, Volovitch M, Prochiantz A, Pierani A (2020) Extracellular Pax6 Regulates Tangential Cajal-Retzius Cell Migration in the Developing Mouse Neocortex. *Cereb Cortex* **30**:465-475 https://doi.org/10.1093/cercor/bhz098 | PubMed

Komai T, Iwanari H, Mochizuki Y, Hamakubo T, Shinkai Y (2009) Expression of the mouse PR domain protein Prdm8 in the developing central nervous system. *Gene Expr Patterns* **9**:503-514 https://doi.org/10.1016/j.gep.2009.07.005 | PubMed

Korenkova O, Liu S, Prlesi I, Pepe A, Albadri S, Del Bene F, Zurzolo C (2025) Tunneling nanotubes enable intercellular transfer in zebrafish embryos. *Dev Cell* **60**:524-534. https://doi.org/10.1016/j.devcel.2024.10.016 | PubMed

Kridsada K, Niu J, Haldipur P, Wang Z, Ding L, Li JJ, Lindgren AG, Herrera E, Thomas GM, Chizhikov VV, *et al.* (2018) Roof Plate-Derived Radial Glial-like Cells Support Developmental Growth of Rapidly Adapting Mechanoreceptor Ascending Axons. *Cell Rep* **23**:2928-2941 https://doi.org/10.1016/j.celrep.2018.05.025 | PubMed

Laumonnerie C, Tong YG, Alstermark H, Wilson SI (2015) Commissural axonal corridors instruct neuronal migration in the mouse spinal cord. *Nat Commun* **6**:7028 https://doi.org/10.1038/ncomms8028 | PubMed

Liebl DJ, Tessarollo L, Palko ME, Parada LF (1997) Absence of sensory neurons before target innervation in brain-derived neurotrophic factor-, neurotrophin 3-, and TrkC-deficient embryonic mice. *J Neurosci* **17**:9113-9121 https://doi.org/10.1523/jneurosci.17-23-09113.1997 | PubMed

Lim L, Mi D, Llorca A, Marin O (2018) Development and Functional Diversification of Cortical Interneurons. *Neuron* **100**:294-313 https://doi.org/10.1016/j.neuron.2018.10.009 | PubMed

Masgutova G, Harris A, Jacob B, Corcoran LM, Clotman F (2019) Pou2f2 Regulates the Distribution of Dorsal Interneurons in the Mouse Developing Spinal Cord. *Front Mol Neurosci* **12**:263 https://doi.org/10.3389/fnmol.2019.00263 | PubMed

Moran-Rivard L, Kagawa T, Saueressig H, Gross MK, Burrill J, Goulding M (2001) Evx1 is a postmitotic determinant of v0 interneuron identity in the spinal cord. *Neuron* **29**:385-399 https://doi.org/10.1016/s0896-6273(01)00213-6 | PubMed

Muller T, Anlag K, Wildner H, Britsch S, Treier M, Birchmeier C (2005) The bHLH factor Olig3 coordinates the specification of dorsal neurons in the spinal cord. *Genes Dev* **19**:733-743 https://doi.org/10.1101/gad.326105 | PubMed

Ohnmacht C, Pullner A, King SB, Drexler I, Meier S, Brocker T, Voehringer D (2009) Constitutive ablation of dendritic cells breaks self-tolerance of CD4 T cells and results in spontaneous fatal autoimmunity. *The Journal of experimental medicine* 549-559 https://doi.org/10.1084/jem.20082394 | PubMed

Panayi H, Panayiotou E, Orford M, Genethliou N, Mean R, Lapathitis G, Li S, Xiang M, Kessaris N, Richardson WD, *et al.* (2010) Sox1 is required for the specification of a novel p2-derived interneuron subtype in the mouse ventral spinal cord. *J Neurosci* **30**:12274-12280 https://doi.org/10.1523/jneurosci.2402-10.2010 | PubMed

Pancho A, Mitsogiannis MD, Aerts T, Dalla Vecchia M, Ebert LK, Geenen L, Noterdaeme L, Vanlaer R, Stulens A, Hulpiau P, *et al.* (2022) Modifying PCDH19 levels affects cortical interneuron migration. *Front Neurosci* **16**:887478 https://doi.org/10.3389/fnins.2022.887478 | PubMed

Park S, Lee C, Sabharwal P, Zhang M, Meyers CL, Sockanathan S (2013) GDE2 promotes neurogenesis by glycosylphosphatidylinositol-anchor cleavage of RECK. *Science* **339**:324-328 https://doi.org/10.1126/science.1231921 | PubMed

Parthasarathy S, Srivatsa S, Nityanandam A, Tarabykin V (2014) Ntf3 acts downstream of Sip1 in cortical postmitotic neurons to control progenitor cell fate through feedback signaling. *Development* **141**:3324-3330 https://doi.org/10.1242/dev.114173 | PubMed

Pelosi B, Migliarini S, Pacini G, Pratelli M, Pasqualetti M (2014) Generation of Pet1210-Cre transgenic mouse line reveals non-serotonergic expression domains of Pet1 both in CNS and periphery. *PLoS One* **9**:e104318 https://doi.org/10.1371/journal.pone.0104318 | PubMed

Peng CY, Yajima H, Burns CE, Zon LI, Sisodia SS, Pfaff SL, Sharma K (2007) Notch and MAML signaling drives Scl-dependent interneuron diversity in the spinal cord. *Neuron* **53**:813-827 https://doi.org/10.1016/j.neuron.2007.02.019 | PubMed

Pfaff SL, Mendelsohn M, Stewart CL, Edlund T, Jessell TM (1996) Requirement for LIM homeobox gene Isl1 in motor neuron generation reveals a motor neuron-dependent step in interneuron differentiation. *Cell* **84**:309-320 https://doi.org/10.1016/s0092-8674(00)80985-x | PubMed

Pillai EK, Franze K (2024) Mechanics in the nervous system: From development to disease. *Neuron* **112**:342-361 https://doi.org/10.1016/j.neuron.2023.10.005 | PubMed

Pineda RH, Svoboda KR, Wright MA, Taylor AD, Novak AE, Gamse JT, Eisen JS, Ribera AB (2006) Knockdown of Nav1.6a Na+ channels affects zebrafish motoneuron development. *Development* **133**:3827-3836 https://doi.org/10.1242/dev.02559 | PubMed

Pipicelli F, Baumann N, Di Giaimo R, Forero-Echeverry A, Kyrousi C, Bonrath R, Maccarrone G, Jabaudon D, Cappello S (2023) Non-cell-autonomous regulation of interneuron specification mediated by extracellular vesicles. *Sci Adv* **9**:eadd8164 https://doi.org/10.1126/sciadv.add8164 | PubMed

Prasad T, Wang X, Gray PA, Weiner JA (2008) A differential developmental pattern of spinal interneuron apoptosis during synaptogenesis: insights from genetic analyses of the protocadherin-gamma gene cluster. *Development* **135**:4153-4164 https://doi.org/10.1242/dev.026807 | PubMed

Renaux E, Baudouin C, Schakman O, Gay O, Martin M, Marchese D, Achouri Y, Rezsohazy R, Gofflot F, Clotman F (2024) Arid3c identifies an uncharacterized subpopulation of V2 interneurons during embryonic spinal cord development. *Front Cell Neurosci* **18**:1466056 https://doi.org/10.3389/fncel.2024.1466056 | PubMed

Rhee HS, Closser M, Guo Y, Bashkirova EV, Tan GC, Gifford DK, Wichterle H (2016) Expression of Terminal Effector Genes in Mammalian Neurons Is Maintained by a Dynamic Relay of Transient Enhancers. *Neuron* **92**:1252-1265 https://doi.org/10.1016/j.neuron.2016.11.037 | PubMed

Riva M, Genescu I, Habermacher C, Orduz D, Ledonne F, Rijli FM, Lopez-Bendito G, Coppola E, Garel S, Angulo MC, *et al.* (2019) Activity-dependent death of transient Cajal-Retzius neurons is required for functional cortical wiring. *eLife* **8** https://doi.org/10.7554/elife.50503 | PubMed

Rousso DL, Gaber ZB, Wellik D, Morrisey EE, Novitch BG (2008) Coordinated actions of the forkhead protein Foxp1 and Hox proteins in the columnar organization of spinal motor neurons. *Neuron* **59**:226-240 https://doi.org/10.1016/j.neuron.2008.06.025 | PubMed

Roy A, Francius C, Rousso DL, Seuntjens E, Debruyn J, Luxenhofer G, Huber AB, Huylebroeck D, Novitch BG, Clotman F (2012) Onecut transcription factors act upstream of Isl1 to regulate spinal motoneuron diversification. *Development* **139**:3109-3119 https://doi.org/10.1242/dev.078501 | PubMed

Sabharwal P, Lee C, Park S, Rao M, Sockanathan S (2011) GDE2 regulates subtype-specific motor neuron generation through inhibition of Notch signaling. *Neuron* **71**:1058-1070 https://doi.org/10.1016/j.neuron.2011.07.028 | PubMed

Sagner A (2024) Temporal patterning of the vertebrate developing neural tube. *Curr Opin Genet Dev* **86**:102179 https://doi.org/10.1016/j.gde.2024.102179 | PubMed

Schnerwitzki D, Perry S, Ivanova A, Caixeta FV, Cramer P, Gunther S, Weber K, Tafreshiha A, Becker L, Vargas Panesso IL, *et al.* (2018) Neuron-specific inactivation of Wt1 alters locomotion in mice and changes interneuron composition in the spinal cord. *Life Sci Alliance* **1**:e201800106 https://doi.org/10.26508/lsa.201800106 | PubMed

Seredick S, Hutchinson SA, Van Ryswyk L, Talbot JC, Eisen JS (2014) Lhx3 and Lhx4 suppress Kolmer-Agduhr interneuron characteristics within zebrafish axial motoneurons. *Development* **141**:3900-3909 https://doi.org/10.1242/dev.105718 | PubMed

Skaggs K, Martin DM, Novitch BG (2011) Regulation of spinal interneuron development by the Olig-related protein Bhlhb5 and Notch signaling. *Development* **138**:3199-3211 https://doi.org/10.1242/dev.057281 | PubMed

Stam FJ, Hendricks TJ, Zhang J, Geiman EJ, Francius C, Labosky PA, Clotman F, Goulding M (2012) Renshaw cell interneuron specialization is controlled by a temporally restricted transcription factor program. *Development* **139**:179-190 https://doi.org/10.1242/dev.071134 | PubMed

Surya A, Sarinay-Cenik E (2022) Cell autonomous and non-autonomous consequences of deviations in translation machinery on organism growth and the connecting signalling pathways. *Open Biol* **12**:210308 https://doi.org/10.1098/rsob.210308 | PubMed

Takahashi M, Sanchez JT (2020) Effects of Neurotrophin-3 on Intrinsic Neuronal Properties at a Central Auditory Structure. *Neurosci Insights* **15** https://doi.org/10.1177/2633105520980442 | PubMed

Teissier A, Griveau A, Vigier L, Piolot T, Borello U, Pierani A (2010) A novel transient glutamatergic population migrating from the pallial-subpallial boundary contributes to neocortical development. *J Neurosci* **30**:10563-10574 https://doi.org/10.1523/jneurosci.0776-10.2010 | PubMed

Toch M, Clotman F (2019) CBP and p300 coactivators contribute to the maintenance of Isl1 expression by the Onecut transcription factors in embryonic spinal motor neurons. *Mol Cell Neurosci* **101**:103411 https://doi.org/10.1016/j.mcn.2019.103411 | PubMed

Toch M, Harris A, Schakman O, Kondratskaya E, Boulland JL, Dauguet N, Debrulle S, Baudouin C, Hidalgo-Figueroa M, Gow A, *et al.* (2020) Onecut-dependent Nkx6.2 transcription factor expression is required for proper formation and activity of spinal locomotor circuits. *Sci Rep* **10**:996 https://doi.org/10.1038/s41598-020-57945-4 | PubMed

Tripodi M, Stepien AE, Arber S (2011) Motor antagonism exposed by spatial segregation and timing of neurogenesis. *Nature* **479**:61-66 https://doi.org/10.1038/nature10538 | PubMed

Usui N, Watanabe K, Ono K, Tomita K, Tamamaki N, Ikenaka K, Takebayashi H (2012) Role of motoneuron-derived neurotrophin 3 in survival and axonal projection of sensory neurons during neural circuit formation. *Development* **139**:1125-1132 https://doi.org/10.1242/dev.069997 | PubMed

Velasco S, Ibrahim MM, Kakumanu A, Garipler G, Aydin B, Al-Sayegh MA, Hirsekorn A, Abdul-Rahman F, Satija R, Ohler U, *et al.* (2017) A Multi-step Transcriptional and Chromatin State Cascade Underlies Motor Neuron Programming from Embryonic Stem Cells. *Cell Stem Cell* **20**:205-217. https://doi.org/10.1016/j.stem.2016.11.006 | PubMed

Vincent C, Gilabert-Juan J, Gibel-Russo R, Alvarez-Fischer D, Krebs MO, Le Pen G, Prochiantz A, Di Nardo AA (2021) Non-cell-autonomous OTX2 transcription factor regulates anxiety-related behavior in the mouse. *Mol Psychiatry* **26**:6469-6480 https://doi.org/10.1038/s41380-021-01132-y | PubMed

von Bartheld CS, Butowt R (2000) Expression of neurotrophin-3 (NT-3) and anterograde axonal transport of endogenous NT-3 by retinal ganglion cells in chick embryos. *J Neurosci* **20**:736-748 https://doi.org/10.1523/jneurosci.20-02-00736.2000 | PubMed

Wan G, Gomez-Casati ME, Gigliello AR, Liberman MC, Corfas G (2014) Neurotrophin-3 regulates ribbon synapse density in the cochlea and induces synapse regeneration after acoustic trauma. *eLife* **3** https://doi.org/10.7554/elife.03564 | PubMed

Welniarz Q, Morel MP, Pourchet O, Gallea C, Lamy JC, Cincotta M, Doulazmi M, Belle M, Meneret A, Trouillard O, *et al.* (2017) Non cell-autonomous role of DCC in the guidance of the corticospinal tract at the midline. *Sci Rep* **7**:410 https://doi.org/10.1038/s41598-017-00514-z | PubMed

Wester JC, Mahadevan V, Rhodes CT, Calvigioni D, Venkatesh S, Maric D, Hunt S, Yuan X, Zhang Y, Petros TJ, *et al.* (2019) Neocortical Projection Neurons Instruct Inhibitory Interneuron Circuit Development in a Lineage-Dependent Manner. *Neuron* **102**:960-975. https://doi.org/10.1016/j.neuron.2019.03.036 | PubMed

Williamson J (2023) Celebrating Spemann-Mangold at 100: An interview with Eddy De Robertis. *Cells Dev* 203828 https://doi.org/10.1016/j.cdev.2023.203828 | PubMed

Wright MA, Ribera AB (2010) Brain-derived neurotrophic factor mediates non-cell-autonomous regulation of sensory neuron position and identity. *J Neurosci* **30**:14513-14521 https://doi.org/10.1523/jneurosci.4025-10.2010 | PubMed

Yamagishi S, Bando Y, Sato K (2020) Involvement of Netrins and Their Receptors in Neuronal Migration in the Cerebral Cortex. *Front Cell Dev Biol* **8**:590009 https://doi.org/10.3389/fcell.2020.590009 | PubMed

Zhang L, Schessl J, Werner M, Bonnemann C, Xiong G, Mojsilovic-Petrovic J, Zhou W, Cohen A, Seeburg P, Misawa H, *et al.* (2008) Role of GluR1 in activity-dependent motor system development. *J Neurosci* **28**:9953-9968 https://doi.org/10.1523/jneurosci.0880-08.2008 | PubMed

Toch M, Clotman F (2019) Expression data from control or conditional Oc-deficient embryonic motor neurons. NCBI Gene Expression Omnibus. ID GSE141949 https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE141949

Martin M (2026) Non-cell_autonomous_regulation_of_spinal_motor_circuit_development_Angla-Navarro-Dominguez-Bajo. GitHub. https://github.com/ManonMartin/Non-cell_autonomous_regulation_of_spinal_motor_circuit_development_Angla-Navarro-Dominguez-Bajo

Peer reviews

Reviewer #1 (Public review):

Summary:

This is a useful study by Angla et al that investigates the basis of observations made from previous studies where loss of Onecut (OC) transcription factors lead to changes in spinal interneuron populations that do not themselves normally express OC. The authors hypothesize that OC expression in spinal motor neurons has non-cell-autonomous effects on pre-motor interneuron (V1, V2a/b/c) population size and distribution. By knocking out OC in the motor neuron lineage (i.e., downstream of Olig2, a motor neuron progenitor marker gene), they indeed show that motor neuron-specific loss of OC expression decreases V2c interneuron number and alters the spatial distribution of V1, V2a, V2b, V2c populations. Using bulk RNA-sequencing of WT and OC conditional knockout (cKO) motor neurons, the authors identify that neurotrophic factor Ntf3 as another factor that is responsible for the non-cell-autonomous effects on the interneuron population; upon knock out of Ntf3 downstream of Olig2 they show that this leads to increased interneuron numbers and alters their spatial distribution, ultimately leading to dysregulation of spinal motor circuits and motor activity.

Strengths:

The authors use sophisticated genetic tools to precisely remove OC and Ntf3 expression in a lineage-specific manner and comprehensively assess the downstream effects across brachial, thoracic, lumbar levels of the spinal cord, as well as at two developmental timepoints, E12.5 and E14.5.

Weaknesses:

The evidence supporting their claims is incomplete. In figure 2 and 4, the control distributions (despite reporting the same populations in the same regions) look different, suggesting large sample-to-sample variance in distribution. The authors explain that this is due to several technical limitations in their study.

Furthermore, the way the Ntf3 results are framed can be misleading; it reads as though one of the main claims of this study is that OC acts through Ntf3.

https://doi.org/10.7554/eLife.110788.2.sa3

Reviewer #2 (Public review):

The study by Angla et al. proposes a model in which NT-3 produced by motor neurons regulates interneuron numbers and distribution in a non-cell autonomous manner. The authors demonstrate that ablation of motor neurons (MNs) and global and conditional deletion of OC transcription factors lead to changes in interneuron distribution. They identify that NT3 is upregulated after MN-specific OC deletion in RNA-seq experiments and show that olig2-cre mediated NT3 deletion leads to increased ventral interneuron numbers, altered distribution and defects in locomotor behavior. The authors conclude that MN-derived NT3, under OC control, regulates interneuron development. While this is an intriguing hypothesis, the study does not consolidate the results from the different experimental models in a cohesive model to provide any mechanistic insights into how NT3 may accomplish this function.

(1) The authors utilize different experimental models (OC transcription factor global and conditional knockouts, DTA ablation and NT3 deletion) to show that MNs may influence interneuron numbers and positioning, but there doesn't seem to be a direct link into how each manipulation relates to the others in an informative way. For example, the relationship between OC and NT3 deletion data is not entirely clear, even though NT3 is downstream of OC transcription factors. Both deletions lead to changes in interneuron distribution but there doesn't seem to be any correlation between the two that relates to relative changes in NT3 levels. Although it is understandable that transcription factor manipulations may have additional effects, it is hard to derive any mechanistic insights into how exactly MNs influence interneuron differentiation or migration from the data presented here. Similarly, while the rationale for following up on NT3 seems to be the chick electroporation experiments, only a very minor decrease in Chx10 interneurons is shown in those experiments. Shouldn't NT3 overexpression lead to substantial decreases in IN numbers according to the authors' model?

(2) The study primarily quantifies interneuron numbers and distribution at different levels of the spinal cord and under different genetic manipulations. In different figures, the values and distributions shown for controls vary quite a lot. For example, in figure 2B vs figure 4B, the number of FoxP2+ V1 neurons at brachial levels is ~350 vs 125. Similarly, the control distributions in 2I and 4I are quite different. While this can be partially explained by technical issues, it remains challenging to determine whether the conclusions regarding the impact of each genetic manipulation on interneuron numbers and distribution are valid.

(3) It is not clear that the behavioral phenotypes seen in the olig2-cre mediated deletion of NT3 can be attributed to specific changes in interneuron development, connectivity or function. While the behavioral phenotype is in theory consistent with defects in motor circuits, there is a big gap between the embryonic changes shown here and behavior, with no in between circuit level changes in locomotor circuits shown. Thus, the behavioral experiments are not very informative as to how exactly components of motor circuits might be altered with the NT3 deletion.

https://doi.org/10.7554/eLife.110788.2.sa2

Reviewer #3 (Public review):

This manuscript aims to investigate cell extrinsic mechanisms that regulate differentiation and distribution of interneuron types in the spinal cord. The authors demonstrate that the loss of motor neurons leads to changes in number and distribution of different interneuron types, specifically V0v, V1, and V2b (but not V2a). The authors then hypothesize that this phenotype may be controlled by the action of Onecut (OC) transcription factors in motor neurons. Conditional knockout of OC1 + OC2 in motor neurons using Olig2-Cre, however, does not lead to significant changes in numbers of V1, V2a, V2b interneurons, although there is a change in their spatial distribution. While the authors do not check V0v neurons in OC mutants, they do check V2c, which show a reduction in number and change in distribution. Why the same neurons are not checked across experiments is unclear. The authors then analyze existing RNA-seq data to identify factors that could be mediating effects of the OC factors in motor neurons. They identify Ntf3 as a candidate and confirm that it is upregulated in OC mutants. Conditional loss of function of Ntf3 (Olig2-Cre) leads to increases in V1, V2a, and V2b (but not V2c) interneurons and changes in distribution of all four interneuron types. However, the Ntf3 phenotypes do not necessarily suggest that it works in the same pathway as OC to control interneuron development. Finally, the authors demonstrate that these Ntf3 conditional mutants have major defects in motor function.

The main conclusions of the manuscript are that i) motor neurons non-cell autonomously control interneuron development, ii) that OC activity in Olig2 lineage cells non-cell autonomously controls aspects of interneuron development, and iii) that Ntf3 in motor neurons non-cell autonomously controls aspects of interneuron development. The mechanistic link between OC and Ntf3 activity is not clear. And the interneuron phenotype seen when motor neurons are depleted it not phenocopied by either loss of OC or Ntf3 from motor neurons. While the authors addressed some of my initial comments, the impact of this work on the field remains difficult to assess for the following reasons:

(1) The manuscript relies heavily on quantifying numbers and spatial distribution of interneuron populations. However, these do not seem to be consistent in control animals across experiments, making it difficult to interpret any changes observed in genetic manipulations. Specifically, in Fig. 2 and 4, the same markers are being used to quantify V1, V2a, V2b, and V2c interneurons in controls vs. OC (Fig. 2) or Ntf3 (Fig. 4) conditional knockouts, but the numbers of neurons and their distribution in control animals are variable between these two figures. For example, there seem to be a mean of >300 V1 neurons in E12.5 brachial sections of Fig. 2 controls, but this number is <150 in Fig. 4 controls. The cell distribution scoring is similarly variable between these controls without any explanation. The same is true for E14.5 controls used in Fig. S2 vs. Fig. S6.

The authors explain in response to the 1st review that technical variability including different antibodies, different researchers performing experiments, and different mouse backgrounds may contribute to this variation, but do not include this explanation clearly in the manuscript where it would be useful for readers. It would have also helped, for example, if authors had labeled datapoints on bar plots based on the animal that they came from to

understand inter-animal variability within experiments. Overall, the biological significance of the results remain difficult to interpret due to high technical variability.

(2) In the initial review, it was noted that there is no data to support the claim that OC1/2 mutants have altered interneuron development because of higher Ntf3 expression. In response to this, the authors note that the manuscript does not claim that OC exerts its non-cell autonomous effects through Ntf3 and add a new paragraph in the discussion which now addresses the fact that it is difficult to compare the phenotypes observed the different genetic manipulations presented in the work. While this paragraph addresses the incomplete mechanistic link between OC and Ntf3, it also decreases the mechanistic impact of the work.

(3) In Figure 3E-3G, the authors show that some V1, V2a, V2b interneurons express the Ntf3 receptor TrkC. It is not stated (apologies if I missed it) what age this expression is, and from the images, it looks like in addition to medially located newborn neurons being negative, laterally located Gata3 interneurons are also broadly negative for TrkC, and many Foxd3 and Chx10 interneurons are also negative. Separate images of each channel and/or scoring would help clarify this. If many relevant interneurons are indeed TrkC negative, how this affects the conclusions should be addressed in the manuscript.

(4) Behavioral phenotypes are not necessarily caused by Ntf3's role in premotor interneuron development.

https://doi.org/10.7554/eLife.110788.2.sa1

Author response:

The following is the authors' response to the original reviews.

> ***Public Reviews:***
>
> ***Reviewer #1 (Public review):***
>
> *Summary:*
>
> *In this study, Angla et al investigate the basis of observations made from previous studies where loss of Onecut (OC) transcription factors leads to changes in spinal interneuron populations that do not themselves normally express OC. The authors hypothesize that OC expression in spinal motor neurons has non-cell-autonomous effects on pre-motor interneuron (V1, V2a/b/c) population size and distribution. By knocking out OC in the motor neuron lineage (i.e., downstream of Olig2, a motor neuron progenitor marker gene), they indeed show that motor neuron-specific loss of OC expression decreases V2c interneuron number and alters the spatial distribution of V1, V2a, V2b, and V2c populations. Using bulk RNA-sequencing of WT and OC conditional knockout (cKO) motor neurons, the authors identify that the neurotrophic factor Ntf3 is downregulated by OC expression. They subsequently hypothesize that the non-cell-autonomous effects observed by loss of OC expression in motor neurons can be explained by de-repression of Ntf3. To test this, the authors conditionally knock out Ntf3 downstream of Olig2 and show that this leads to increased interneuron numbers and alters their spatial distribution, ultimately leading to dysregulation of spinal motor circuits and motor activity.*
>
> *Strengths:*
>
> *The authors use sophisticated genetic tools to precisely remove OC and Ntf3 expression in a lineage-specific manner and comprehensively assess the downstream effects across brachial, thoracic, lumbar levels of the spinal cord, as well as at two developmental timepoints, E12.5 and E14.5.*

> *Weaknesses:*
>
> *There are two main concerns that are not fully addressed:*
>
> *(1) Based on the effects observed with OC vs. Ntf3 cKO, it is unclear whether OC is indeed exerting its non-cell-autonomous effects via Ntf3. Knocking out both Ntf3 and OC and comparing the effects to those seen with just OC cKO alone could provide more insight on this point.*

In this study, we did not intend to demonstrate that Onecut transcription factors exert their non-cell autonomous action on spinal interneuron development by regulating Ntf3 expression, and we do not state in the manuscript that this is the case. We only show that Onecut factors and Ntf3, the expression of which they regulate, contribute to the non-cell autonomous regulation of spinal interneuron development by the motor neurons. We are convinced that Onecut factors could regulate multiple independent factors and pathways involved in extrinsic regulation of interneuron development. This possibly also includes, as demonstrated in cell culture for multiple homeoproteins including human Onecut factors, the intercellular transfer of the Onecut homeoproteins during spinal cord development, a process that we are currently investigating. Knocking out both OC and Ntf3 in the motor neurons, beyond being technically extremely challenging (1/64 probability to obtain double-mutant embryos), would not enable to address this question, as it will simply results in the addition of two different defects.

> *Also, a quantitative summary of the effects of Ntf3 overexpression in motor neurons in the chick is lacking.*

A quantitative summary of the effects of Ntf3 overexpression in the chicken embryonic spinal cord is provided in Figure S2.

> *(2) How the authors assess changes in the spatial distribution of interneurons is unclear. In Figures 2 and 4, the control distributions (despite reporting the same populations in the same regions) look different, suggesting large sample-to-sample variance in distribution. Although the authors report that several sections in each level were taken from at least three animals for each condition, it's unclear how variance within WT or cKO sections was accounted for in the final statistical evaluation. It seems at a glance that a comparison between control samples in Figure 2 and Figure 4 could report statistically significant differences, which would be problematic. A more rigorous report of sample-to-sample variance and a more in-depth explanation of the statistical methods are needed.*

The experimental procedure to analyze the spatial distribution of spinal interneurons at different stages of development is described in details in the “Statistical analyses” paragraph of the Materials and Methods section of the manuscript, and has been repeatedly used by ourselves [1,2] and by others (see for example [3-5]) to conduct similar analyses.

We also noticed that the distribution of the different analyzed interneuron populations in the control embryos showed some differences between the *cOc1Oc2*$^{-/-}$ and in the *cNtf3*$^{-/-}$ lines. Several parameters can account for this observation. First, this study has been conducted over a period of 15 years, different investigators each contributing to different steps of the analysis. Second, the genetic background of these two lines is not identical, impacting both the duration of the gestation (hence, the embryonic stage of the performed analyses, even if the embryos were collected on the same gestation day) and possibly the distribution of some interneuron populations. Third, because of evolutions in the availability of the primary antibodies used to label the interneuron populations of interest, the same antibodies were not used throughout the study, as stated for in the Materials and Methods section for Chx10,

Gata3 and Sox1, although the same antibody was used by the same investigator to label the same interneuron population in each mouse line at each developmental stage.

A detailed description of the number of sections and embryos included in each analysis as well as the whole statistical workflow that was used for the distribution analyses, which takes into account variance within control or mutant samples, is now provided in the revised version of the manuscript.

> ***Reviewer #2 (Public review):***
>
> *The study by Angla et al. proposes a model in which NT-3 produced by motor neurons regulates interneuron numbers and distribution in a non-cell autonomous manner. The authors demonstrate that ablation of motor neurons (MNs) and global and conditional deletion of OC transcription factors lead to changes in interneuron distribution. They identify that NT3 is upregulated after MN-specific OC deletion in RNA-seq experiments and show that olig2-cre mediated NT3 deletion leads to increased ventral interneuron numbers, altered distribution, and defects in locomotor behavior. The authors conclude that MN-derived NT3, under OC control, regulates interneuron development. While this is an intriguing hypothesis, additional experiments are needed to support it and strengthen the link between the different experiments described here.*
>
> *(1) The study primarily quantifies interneuron numbers and distribution at different levels of the spinal cord and under different genetic manipulations. Experimental details are lacking, defining how many sections were analyzed (several are noted in the methods) and how the rostrocaudal levels of the spinal cord were precisely aligned.*

A detailed description of the number of sections and embryos included in each analysis as well as the whole statistical workflow that was used for the distribution analyses is now provided in the revised version of the manuscript. The rostrocaudal levels of the spinal cord were precisely aligned using the distribution of Foxp1 in the Lateral Motor Columns (LMCs) at brachial or lumbar levels of the spinal cord, which is also indicated in the revised version.

> *In different figures, the values and distributions shown for controls vary quite a lot. For example, in Figure 2B vs Figure 4B, the number of FoxP2+ V1 neurons at brachial levels is ~350 vs 125. Similarly, the control distributions in 2I and 4I are quite different. This makes it challenging to determine whether the conclusions regarding the impact of each genetic manipulation on interneuron numbers and distribution are valid.*

Several parameters can account for these observations. First, this study has been conducted over a period of 15 years, different investigators each contributing to different steps of the analysis. Second, the genetic background of these two lines is not identical, impacting both the duration of the gestation (hence, the embryonic stage of the performed analyses, even if the embryos were collected on the same gestation day) and possibly the distribution of some interneuron populations. Third, because of evolutions in the availability of the primary antibodies used to label the interneuron populations of interest, the same antibodies were not used throughout the study, as stated for in the Materials and Methods section for Chx10, Gata3 and Sox1, although the same antibody was used by the same investigator to label the same interneuron population in each mouse line at each developmental stage.

> *(2) The relationship between OC and NT3 deletion data is not entirely clear. Both deletions presumably lead to changes in interneuron distribution, but is there any reverse relationship between the two that relates to relative changes in NT3 levels? The authors do not directly compare NT3 and OC KO IN distributions. Similarly, one might expect a decrease in interneuron numbers in OC mutants, which is only reported for V2c neurons. However, the image presented in Figure 2G shows an equal number of V2c INs in control and mutant.*

In this study, we did not intend to demonstrate that Onecut transcription factors exert their non-cell autonomous action on spinal interneuron development by regulating Ntf3 expression, and we do not state in the manuscript that this is the case. We only show that Onecut factors and Ntf3, the expression of which they regulate, contribute to the non-cell autonomous regulation of spinal interneuron development by the motor neurons. We are convinced that Onecut factors could regulate multiple independent factors and pathways involved in extrinsic regulation of interneuron development. For example, as mentioned above, we observed intercellular transfer of the Onecut homeoproteins during spinal cord development, suggesting different strategies for non-cell autonomous regulation of interneuron development. The two mouse lines studied here consist on the one side in a combination of OC inactivation and Ntf3 increased expression, and on the other side in Ntf3 inactivation. Therefore, a reverse relationship between the changes in interneuron distribution is not expected. Furthermore, gain-of-function and loss-of-function experiments in mouse models frequently generate phenotypes that are not inverse to each other.

> *(3) It is not clear that the behavioral phenotypes seen in the olig2-cre mediated deletion of NT3 can be attributed to changes in interneuron development. How about a role of NT3 in oligodendrocytes? There is a big gap between the embryonic changes shown here and behavior, with no in-between circuit-level changes in locomotor circuits shown.*

We agree, the motor behavior changes that we recorded in Ntf3 conditional mutant mice are, as stated, “consistent with the hypothesis that Ntf3 produced by MNs is required to generate locomotor circuits with properly coordinated activity” but do not demonstrate a direct causal relationship. However, investigating the intrinsic activity of the spinal locomotor circuits, independently from, for example, oligodendrocyte contribution may prove to be extremely challenging and was beyond the scope of this study. In addition, to our best knowledge, *Ntf3* has not been shown to be expressed in healthy oligodendrocytes *in vivo*, and TrkC has not been reported to be displayed by these cells in the same conditions.

> *A more restricted manipulation would be deleting TrkC from specific interneuron populations. Related to this, although TrkC is shown to be broadly expressed in ventral interneurons, it is not shown specifically to colocalize with any of the interneuron markers. The authors should validate that the receptor is expressed in the subsets that they are investigating.*

We agree, investigating the consequences of inactivating the TrkC receptor in specific interneuron populations would be extremely informative. However, this experiment is also very challenging to perform, as most of the driver lines available to target spinal interneuron populations additionally target multiple neuronal populations outside of the spinal cord that are also involved in the control of movements and could therefore induce confounding effects on motor behavior analyses.

We thank the reviewer for suggesting to investigate in more details the interneuron populations that display TrkC receptors, which is now included in the revised version of the manuscript (Figure 3E-G).

> *(4) The rationale for following up on NT3 seems to be the chick electroporation experiments; however, no changes in distribution are shown in those experiments, and only a very minor decrease in Chx10 interneurons. Shouldn't NT3 overexpression lead to substantial decreases in IN numbers according to the authors' model? The "data not shown", which presumably refers to distribution, would be important to show here, to further support this rationale.*

Chicken spinal cord electroporation only enables to study spinal cord development for a few days, given the high mortality rate observed after longer incubation. At the stage we collected

the electroporated embryos for analyses, interneuron migration has barely been initiated, and distribution cannot be studied yet. Consistently, we are not aware of any report of interneuron distribution analysis in electroporated chicken embryonic spinal cord, as compared to mouse embryos [1-5].

> *(5) The idea that NT3 downregulation causes an increase in IN numbers is not intuitive. Also, considering the DTA experiments in Figure 1, showing that MN ablation leads to a decrease in several IN subtypes and no changes in V2a neurons. It would be helpful for the reader if the authors could synthesize their results in the discussion and reconcile their experimental findings.*

We agree, this has now been included in the Discussion section of the revised manuscript.

> ***Reviewer #3 (Public review):***
>
> *This manuscript aims to investigate cell extrinsic mechanisms that regulate the differentiation and distribution of interneuron types in the spinal cord. The authors demonstrate that the loss of motor neurons leads to changes in the number and distribution of different interneuron types, specifically V0v, V1, and V2b (but not V2a). The authors then hypothesize that this phenotype may be controlled by the action of Onecut (OC) transcription factors in motor neurons. Conditional knockout of OC1 + OC2 in motor neurons using Olig2-Cre, however, does not lead to significant changes in the numbers of V1, V2a, and V2b interneurons, although there is a change in their spatial distribution. While the authors do not check V0v neurons in OC mutants, they do check V2c, which show a reduction in number and change in distribution. Why the same neurons are not checked across experiments is unclear. The authors then analyze existing RNA-seq data to identify factors that could be mediating the effects of the OC factors in motor neurons. They identify Ntf3 as a candidate and confirm that it is upregulated in OC mutants. Conditional loss of function of Ntf3 (Olig2-Cre) leads to increases in V1, V2a, and V2b (but not V2c) interneurons and changes in the distribution of all four interneuron types. Finally, the authors demonstrate that these Ntf3 conditional mutants have major defects in motor function.*
>
> *The conclusions of this manuscript are not well supported by the data for the reasons listed below, making it difficult to assess the impact of this work on the field.*
>
> *(1) The manuscript relies heavily on quantifying numbers and the spatial distribution of interneuron populations. However, these do not seem to be consistent in control animals across experiments, making it difficult to interpret any changes observed in genetic manipulations. Specifically, in Figures 2 and 4, the same markers are being used to quantify V1, V2a, V2b, and V2c interneurons in controls vs. OC (Figure 2) or Ntf3 (Figure 4) conditional knockouts, but the numbers of neurons and their distribution in control animals are variable between these two figures. For example, there seems to be a mean of >300 V1 neurons in E12.5 brachial sections of Fig. 2 controls, but this number is <150 in Fig. 4 controls. The cell distribution scoring is similarly variable between these controls without any explanation. The same is true for E14.5 controls used in Figure S1 vs. Figure S3.*

We also noticed that quantifications and distributions of the different analyzed interneuron populations in the control embryos showed some differences between the *cOc1Oc2*$^{-/-}$ and in the *cNtf3*$^{-/-}$ lines. Several parameters can account for this observation. First, this study has been conducted over a period of 15 years, different investigators each contributing to different steps of the analysis. Therefore, the interneuron distribution analyses in these two mouse lines were not conducted by the same researchers. Second, the genetic background of these two lines is not identical, impacting both the duration of the gestation (hence, the embryonic stage of the performed analyses, even if the embryos were collected on the same

gestation day) and possibly the distribution of some interneuron populations. Third, because of evolutions in the availability of the primary antibodies used to label the interneuron populations of interest, the same antibodies were not used throughout the study, as stated in the Materials and Methods section for Chx10, Gata3 and Sox1, although the same antibody was used by the same investigator to label the same interneuron population in each mouse line at each developmental stage.

> *(2) Neurotrophic factors generally promote neuronal survival. However, in this study, the loss of Ntf3 leads to increased numbers of interneurons. This finding is in disagreement with previous observations in slice cultures of spinal cords, as stated in the discussion. This discrepancy makes it even more important that the cell counts reported in the figures discussed above are robust.*

Considering that neurotrophic factors only support neuronal survival would strongly neglect their important function in neuronal differentiation, which has been broadly demonstrated. Severe immunotoxic ablation of motor neurons or anti-serum blockade of Ntf3 activity severely depleted inhibitory, but not excitatory, interneurons in a highly apoptotic-prone organotypic culture model of embryonic rat spinal cord slices, which was rescued by Ntf3 in the first model [6]. Opposite results were obtained *in vivo* by other researchers using mouse models lacking almost all MNs due to the elimination of skeletal muscles, where the number of spinal INs remained unaffected [7,8]. Combined to our results, these *in vivo* observations suggest that Ntf-3 is involved in interneuron differentiation rather in their survival. Consistently, Ntf3 has been shown to promote neuronal differentiation [9].

> *(3) The claim that phenotypes are non-cell autonomously driven by motor neurons is not well supported. In Olig2-Cre conditional knockouts of Onecut and Ntf3, there is no confirmation that the loss of these factors is specific to motor neurons. Therefore, it cannot be ruled out that other cell populations may be mediating the phenotypes.*

Combined conditional inactivation of Oc1 and Oc2 has been reported in [10]. Conditional inactivation of *Ntf3* only impacts motor neurons as it is the only cell population in the ventral spinal cord wherein this factor is produced (this study and [11-13]). Furthermore, Olig2-Cre has been shown to be active in motor neurons and in V3 interneurons (see for example [14], which, for this reason, have not been studied in the frame of this project as stated in the manuscript.

> *(4) The claim that interneuron development is regulated by OC control of Ntf3 expression in motor neurons is not well supported. The authors show that loss of OC1/2 leads to an increase in Ntf3 expression in motor neurons. If this pathway were controlling interneurons, loss of OC function and overexpression of Ntf3 would have the same phenotype, which is not the case. Additionally, it would also be expected that loss of OC function and loss of Ntf3 function would have inverse phenotypes, which is also not the case. The phenotypes from OC loss of function and Ntf3 loss of function seem distinct from one another. The authors state that too little and too much Ntf3 are both bad for interneuron development, but there is no data to support their claim that OC1/2 mutants have altered interneuron development because of higher Ntf3 expression.*

In this study, we did not intend to demonstrate that Onecut transcription factors exert their non-cell autonomous action on spinal interneuron development by regulating Ntf3 expression, and we do not state in the manuscript that this is the case. We only show that Onecut factors and Ntf3, the expression of which they regulate, contribute to the non-cell autonomous regulation of spinal interneuron development by the motor neurons. We are convinced that Onecut factors could regulate multiple independent factors and pathways involved in extrinsic regulation of interneuron development. This possibly also includes, as demonstrated in cell culture for multiple homeoproteins including human Onecut factors, the

intercellular transfer of the Onecut homeoproteins during spinal cord development, a process that we are currently investigating.

> *(5) It is not clear that interneurons being studied express the Ntf3 receptor TrkC, which makes it difficult to assess whether changes in Ntf3 signaling are directly responsible for the phenotype.*

We thank the reviewer for suggesting to investigate in more details the interneuron populations that display TrkC receptors, which is now included in the revised version of the manuscript (Figure 3E-G).

> *(6) While the behavioral phenotypes are consistent with Ntf3 playing a role in motor circuits, there is no evidence to suggest that Ntf3's influence on premotor interneurons being studied is driving or contributing to this phenotype, as discussed by the authors.*

We agree, the motor behavior changes that we recorded in Ntf3 conditional mutant mice are, as stated, "consistent with the hypothesis that Ntf3 produced by MNs is required to generate locomotor circuits with properly coordinated activity" but do not demonstrate a direct causal relationship. However, investigating the intrinsic activity of the spinal locomotor circuits was beyond the scope of this study.

> ***Recommendations for the authors:***
>
> ***Reviewer #1 (Recommendations for the authors):***
>
> *(1) Figure 3: A more comprehensive overview of the RNA-seq results would be helpful to see how much gene expression is shifted as a result of OC loss (e.g., volcano plot, pathway enrichment analysis).*

We agree, this has now been added in Figures 3B and S3 of the revised manuscript.

> *(2) Figure 2, 4: How does the spatial distribution of motor neurons change?*

Describing the distribution of motor neurons is highly challenging given that the expression of markers that are collectively required to label all the motor neuron subset, including Foxp1, Hb9 and most significantly Isl1, is altered when Onecut expression is modified. Furthermore, changes in motor neuron distribution could constitute consequences of alterations in pre-motor interneuron numbers or distributions. However, as far as we can observe, motor neuron distribution was not obviously modified in the studied mutants.

> *(3) Figure S2: Why is Ntf3 expression so limited compared to Isl1-expressing motor neurons? An explanation would be helpful.*

As stated in the Material and Methods section and in the legend of Figure S4 of the revised manuscript, expression after chicken embryonic spinal cord electroporation is transient and maximal reporter gene expression, including *GFP*, can usually only be visualized 24h after electroporation, but not at later stages. Forty-height hours after electroporation, only residual expression can still be detected in a few targeted cells.

> ***Reviewer #3 (Recommendations for the authors):***
>
> *(1) Changes in interneuron numbers are attributed to defects in differentiation. However, the possibility that these could be caused by changes in cell death has not been ruled out.*

Absence of increased cell death had been demonstrated previously for Onecut mutant embryos [15] and has been verified now in conditional *Ntf3* mutant embryos (Figure S5). Both information have been included in the text.

> *(2) In Figures 2 and 4, it is unclear if the images of the spinal cords are from brachial, thoracic, or lumbar levels. As the three levels frequently show different phenotypes, it is difficult to match the immunostaining with the reported quantification.*

All the pictures show thoracic regions of the spinal cord, as now stated in the figure legends of the revised version of the manuscript.

> *(3) Statistics are missing in the cell distribution plots in Figure S1.*

This is not the case in our version of the pdf file.

> *(4) In experiments where motor neurons are eliminated with Olig2-Cre/DTA, the authors write that progenitors are not affected without showing data. These data should be included because i) Olig2 is expressed in progenitors, so it would be expected that motor neuron progenitors are lost or reduced in this mouse, and ii) showing no changes in progenitors is crucial to the claim that postmitotic motor neurons are affected interneuron populations.*

We agree, these data are now included as Figure S1 of the revised version of the manuscript.

(1) Harris, A. et al. Onecut factors and Pou2f2 regulate the distribution of V2 interneurons in the mouse developing spinal cord. Front Cell Neurosci 13 (2019). *https://doi.org/10.3389/fncel.2019.00184*

(2) Kabayiza, K. U. et al. The Onecut Transcription Factors Regulate Differentiation and Distribution of Dorsal Interneurons during Spinal Cord Development. Front Mol Neurosci 10, 157 (2017). *https://doi.org/10.3389/fnmol.2017.00157*

(3) Deska-Gauthier, D. et al. Embryonic temporal-spatial delineation of excitatory spinal V3 interneuron diversity. Cell Rep 43, 113635 (2024). *https://doi.org/10.1016/j.celrep.2023.113635*

(4) Bikoff, J. B. et al. Spinal Inhibitory Interneuron Diversity Delineates Variant Motor Microcircuits. Cell 165, 207–219 (2016). *https://doi.org/10.1016/j.cell.2016.01.027*

(5) Hayashi, M. et al. Graded Arrays of Spinal and Supraspinal V2a Interneuron Subtypes Underlie Forelimb and Hindlimb Motor Control. Neuron 97, 869–884 e865 (2018). *https://doi.org/10.1016/j.neuron.2018.01.023*

(6) Bechade, C., Mallecourt, C., Sedel, F., Vyas, S. & Triller, A. in J Neurosci Vol. 22 8779–8784 (2002).

(7) Grieshammer, U., Lewandoski, M., Prevette, D., Oppenheim, R. W. & Martin, G. R. Muscle-specific cell ablation conditional upon Cre-mediated DNA recombination in transgenic mice leads to massive spinal and cranial motoneuron loss. Dev Biol 197, 234–247 (1998). *https://doi.org/10.1006/dbio.1997.8859*

(8) Kablar, B. & Rudnicki, M. A. Development in the absence of skeletal muscle results in the sequential ablation of motor neurons from the spinal cord to the brain. Dev Biol 208, 93–109 (1999). *https://doi.org/10.1006/dbio.1998.9184*

(9) Dutton, R., Yamada, T., Turnley, A., Bartlett, P. F. & Murphy, M. Regulation of spinal motoneuron differentiation by the combined action of Sonic hedgehog and neurotrophin 3. Clin Exp Pharmacol Physiol 26, 746–748 (1999). *https://doi.org/10.1046/j.1440-1681.1999.03108.x*

(10) Toch, M. et al. Onecut-dependent Nkx6.2 transcription factor expression is required for proper formation and activity of spinal locomotor circuits. Sci Rep 10, 996 (2020).

https://doi.org/10.1038/s41598-020-57945-4

(11) Buck, C. R., Seburn, K. L. & Cope, T. C. Neurotrophin expression by spinal motoneurons in adult and developing rats. J Comp Neurol 416, 309–318 (2000). *https://doi.org/10.1002/(sici)1096-9861(20000117)416:3<309::aid-cne3>3.0.co;2-u*

(12) Henderson, C. E. et al. Neurotrophins promote motor neuron survival and are present in embryonic limb bud. Nature 363, 266–270 (1993). *https://doi.org/10.1038/363266a0*

(13) Usui, N. et al. Role of motoneuron-derived neurotrophin 3 in survival and axonal projection of sensory neurons during neural circuit formation. Development 139, 1125–1132 (2012). *https://doi.org/10.1242/dev.069997*

(14) Debrulle, S. et al. Vsx1 and Chx10 paralogs sequentially secure V2 interneuron identity during spinal cord development. Cell Mol Life Sci 77, 4117–4131 (2020). *https://doi.org/10.1007/s00018-019-03408-7*

(15) Roy, A. et al. Onecut transcription factors act upstream of Isl1 to regulate spinal motoneuron diversification. Development 139, 3109–3119 (2012). *https://doi.org/10.1242/dev.078501*

https://doi.org/10.7554/eLife.110788.2.sa0