

# Identification of regulators of V2 interneuron diversification and migration during spinal cord development

Thesis submitted for the degree of Doctor of Philosophy  
in Biomedical and Pharmaceutical Sciences

Audrey Harris

Promoter: Pr. Frédéric Clotman





Je tiens à adresser mes remerciements à tous ceux qui m'ont apporté soutien, encouragement et conseil durant cette thèse de doctorat.

Tout d'abord, je remercie mon promoteur, le professeur Frédéric Clotman, pour m'avoir donné l'opportunité de réaliser une thèse dans son laboratoire, pour ses conseils, pour sa patience et sa disponibilité même après avoir frappé pour la dixième fois à la porte de son bureau (en seulement 1 heure). Merci de m'avoir fait confiance.

Que serait cette aventure sans la rencontre de merveilleuses personnes pour vous accompagner tout au long de ce parcours. Je tiens à remercier chaleureusement tous les petits NEDI qui ont souvent rendu les choses plus faciles. Merci à Mathilde de m'avoir fait confiance durant son mémoire (cordon forever), pour nos fou-rires, son soutien et ses encouragements sans faille, à Steph pour sa bonne humeur communicative, sa motivation et d'avoir été la meilleure office-mate ever, à Gauhar pour son aide, ses encouragements et de m'avoir souvent comprise étant dans la même barque que moi, à ChaCha d'être ce petit suricate qu'on aime et pour sa joie de vivre, à Vincent d'avoir égaillé le labo en poussant la chansonnette. Merci à Amandine pour son aide au cours de ce projet et sa disponibilité, à Barbara et Maria d'avoir apporté cette énergie, cette bonne humeur latine et pour tous leurs conseils, à Romélia pour ton aide précieuse et nos toutes nos discussions. Merci aussi à Karolina, Alexia, Cédric, Thibault, Emilie et les Agnès.

Je remercie de tout cœur mes parents et ma sœur d'avoir toujours cru en moi, de m'avoir soutenu et fait confiance même quand la route n'était pas toute tracée. Merci de vous être toujours inquiétés et fait en sorte que tout se passe au mieux pour moi durant mes études et ma thèse. Merci pour tout.

Un immense merci à Jérémy d'avoir embarquer pour l'aventure « thèse » avec moi, de m'avoir si souvent soutenu, encouragé, supporté et d'avoir cru en moi au cours de ce long parcours parsemé de surprises. Ton amour, ta présence et ta compréhension ont été d'une aide inestimable. Merci à Marie-Paule, Delphine, Renaud, Audrey et Vincent pour votre soutien.

J'ai aussi pu compter sur l'interminable soutien de ma meilleure amie, Julie. Merci de m'avoir si souvent pris la main et d'avoir eu les mots juste pour me dire que j'arriverais au bout. Tes encouragements, ta fierté, ta motivation ont été précieux. Merci aussi à Amandine, Alice, Auré, Mayt, Ludy et Emilie d'être des supers amies sur qui on peut compter.

Je ne peux bien sûr pas oublier cette belle famille SBIMs qui ne cesse de s'agrandir. Merci à Auré, Marie (Roux), Laura, Lorraine, Guillaume, Gaëlle, Matthieu, Géraldine, Laurence et Marie d'être ces amis avec qui on passe toujours un merveilleux moment et avec qui j'ai tellement ri pendant mes études.

## Summary

The locomotion is a complex behavior coordinated by Central Pattern Generators located in the ventral region of the spinal cord. These circuits are composed of motor neurons, and of dorsal or ventral interneurons. The ventral interneurons initially constitute 4 distinct cardinal populations, called V0, V1, V2 and V3. Each interneuron population diversifies and migrates to a precise location within the spinal parenchyma in order to properly integrate into the spinal neuronal circuits. Recent studies demonstrated that interneuron diversification and position are key determinants for circuit formation. However, the molecular mechanisms that control those processes remain poorly characterized. Recent data obtained in our laboratory have shown that the Onecut (OC) transcription factors, namely hepatocyte nuclear factor-6 (HNF-6), OC-2 and OC-3 are present in several subsets of ventral interneuron populations. This suggests that they could contribute to the regulation of the ventral interneuron diversification and migration. The aim of my thesis project is to study the roles of the OC factors during these processes and to elucidate the underlying molecular mechanisms.

In this work, we provide evidence that the ventral interneuron populations diversify in multiple subpopulations regionally distributed along the antero-posterior axis of the spinal cord. We show that the OC transcription factors are necessary for proper diversification and migration of the spinal V2 interneurons. Moreover, we highlight that the OC factors control the expression of spinal *Pou2f2* isoforms and of *Nkx6.2*. By gain- or loss-of-function experiments, we show that *Pou2f2* and *Nkx6.2* contribute to the regulation of the V2 distribution.

Thus, we uncover genetic cascades regulate V2 interneuron migration during embryonic development.



## Abbreviation list

|               |                                              |
|---------------|----------------------------------------------|
| BDNF          | Brain derived neurotrophic factor            |
| BMP           | Bone Morphogen protein                       |
| CBP           | CREB-binding protein                         |
| CNS           | Central nervous system                       |
| CPG           | Central pattern generator                    |
| Disp1         | Dispatched1                                  |
| Dll4          | Delta-like4                                  |
| DRG           | dorsal root ganglia                          |
| e             | Embryonic stage                              |
| En1           | Engrailed-1                                  |
| FGF           | Fibroblast Growth Factor                     |
| GDNF          | Glial-derived neurotrophic factor            |
| H3K4me3       | Histone H3-lysine4 trimethylation            |
| HD            | Homeodomain                                  |
| HMC           | Hypaxial motor column                        |
| HNF-6         | Hepatocyte nuclear factor-6                  |
| HNF-3 $\beta$ | Hepatocyte nuclear factor-3 $\beta$          |
| HxRE          | Hexamer response element                     |
| IN            | Interneuron                                  |
| LC            | Locus Coeruleus                              |
| LMC           | Lateral motor column                         |
| LMCI          | Lateral division of the Lateral motor column |
| LMCm          | Medial division of the Lateral motor column  |
| mdDA          | Meso-diencephalic dopaminergic               |
| miR           | Micro-RNA                                    |
| MMC           | Medial motor column                          |
| MN            | Motoneuron                                   |
| MTN           | Mesencephalic trigeminal nucleus             |
| Nicd          | Notch intracellular domain                   |
| NMJ           | Neuromuscular junction                       |
| NRG           | Neuregulin                                   |
| OC            | Onecut                                       |
| Oct-2         | Octamer protein-2                            |

|       |                                      |
|-------|--------------------------------------|
| p     | Progenitors                          |
| p/CAF | p300/CBP-associated factor           |
| pMN   | Motoneuron progenitor                |
| PGC   | Preganglionic column                 |
| Ptc1  | Patched1                             |
| RA    | Retinoic acid                        |
| RC    | Renshaw cell                         |
| Sema  | Semphorin                            |
| Shh   | Sonic Hedgehog                       |
| Smo   | Smoothened                           |
| Ssdp  | Single-stranded DNA-binding proteins |
| TeRE  | Tetramer response element            |
| TTR   | Transthyretin                        |
| VLF   | Ventrolateral funiculus              |

## Table of contents

|                                                                                                                                          |           |
|------------------------------------------------------------------------------------------------------------------------------------------|-----------|
| <b>Foreword</b>                                                                                                                          | <b>11</b> |
| <b>1. Introduction</b>                                                                                                                   | <b>13</b> |
| 1.1. <i>Spinal cord regionalization</i>                                                                                                  | 13        |
| 1.1.1. Antero-posterior patterning of the developing spinal cord                                                                         | 13        |
| 1.1.2. Dorsal-ventral patterning of the spinal cord                                                                                      | 14        |
| 1.1.3. Ventral patterning mediated by Shh                                                                                                | 14        |
| 1.2. <i>Ventral neuronal circuits</i>                                                                                                    | 17        |
| 1.2.1. Motor neurons                                                                                                                     | 18        |
| 1.2.2. Commissural interneurons                                                                                                          | 21        |
| V0 interneurons                                                                                                                          | 22        |
| V3 interneurons                                                                                                                          | 24        |
| 1.2.3. Non commissural interneurons                                                                                                      | 25        |
| V1 interneurons                                                                                                                          | 25        |
| V2 interneurons                                                                                                                          | 27        |
| 1.3. <i>Diversification and migration of spinal IN populations</i>                                                                       | 35        |
| 1.4. <i>The Onecut transcription factors</i>                                                                                             | 39        |
| 1.4.1. Onecut factor structure                                                                                                           | 40        |
| 1.4.2. Transcriptional activity                                                                                                          | 41        |
| 1.4.3. Pattern of expression and roles of OC factors                                                                                     | 44        |
| <b>2. Objectives and strategies</b>                                                                                                      | <b>57</b> |
| <b>3. Results</b>                                                                                                                        | <b>59</b> |
| 3.1. <i>Identification of subsets of ventral interneurons and distribution along the rostrocaudal axis of the developing spinal cord</i> | 59        |
| 3.1.1. Foreword                                                                                                                          | 59        |
| 3.1.2. Introduction                                                                                                                      | 62        |
| 3.1.3. Results                                                                                                                           | 67        |
| Ventral spinal INs are differentially distributed along the rostrocaudal axis of the spinal cord.                                        | 67        |
| Ventral spinal INs can be subdivided into subsets with distinct molecular identity                                                       | 70        |
| Homogenous subsets of V0 INs along the rostrocaudal axis of the spinal cord                                                              | 71        |
| V1 interneuron subsets are differentially distributed along the rostrocaudal axis of the developing spinal cord                          | 74        |
| V2 interneuron subsets are homogeneous along the rostrocaudal axis of the spinal cord                                                    | 78        |
| Differential distribution of V3 interneuron subsets along the rostrocaudal axis of the spinal cord.                                      | 83        |
| 3.1.4. Discussion                                                                                                                        | 89        |
| Topographic and columnar organization of ventral spinal INs.                                                                             | 89        |



|                                                                                                                                                                    |            |
|--------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------|
| <i>Control of the diversification of ventral INs</i>                                                                                                               | 91         |
| 3.1.5. Conclusion and perspectives.                                                                                                                                | 94         |
| 3.1.6. Materials and Methods                                                                                                                                       | 96         |
| 3.1.7. Acknowledgments                                                                                                                                             | 99         |
| 3.1.8. Supplementary figures                                                                                                                                       | 103        |
| 3.2. <i>Onecut factors and Pou2f2 regulate diversification and migration of V2 interneurons in the developing spinal cord</i>                                      | 107        |
| 3.2.1. Foreword                                                                                                                                                    | 107        |
| The Pou2f2 transcription factor                                                                                                                                    | 109        |
| 3.2.2. Introduction                                                                                                                                                | 117        |
| 3.2.3. Results                                                                                                                                                     | 120        |
| OC factors are present in multiple subsets of spinal V2 interneurons                                                                                               | 120        |
| OC factors regulate the diversification of spinal V2 interneurons                                                                                                  | 122        |
| OC factors regulate the distribution of spinal V2 interneurons                                                                                                     | 126        |
| Pou2f2-positive V2 interneurons are mislocated in the absence of OC factors                                                                                        | 135        |
| Pou2f2 regulates the distribution of spinal V2 interneurons                                                                                                        | 138        |
| 3.2.4. Discussion                                                                                                                                                  | 142        |
| Control of V2 IN diversification by the OC factors                                                                                                                 | 142        |
| Control of V2 IN migration by the OC factors                                                                                                                       | 145        |
| A OC-Pou2f2 genetic cascade regulates the migration of V2 INs                                                                                                      | 147        |
| 3.2.5. Materials and methods                                                                                                                                       | 150        |
| 3.2.6. Acknowledgements                                                                                                                                            | 155        |
| 3.2.7. Supplementary Figures                                                                                                                                       | 156        |
| 3.3. <i>Onecut factors control the expression of Nkx6.2, which contributes to the control of the distribution of V2 interneurons in the developing spinal cord</i> | 160        |
| 3.3.1. Foreword                                                                                                                                                    | 160        |
| 3.3.2. Introduction                                                                                                                                                | 160        |
| 3.3.3. Results                                                                                                                                                     | 163        |
| OC factors control the expression of Nkx6.2                                                                                                                        | 163        |
| Nkx6.2 is expressed in the V2a interneurons                                                                                                                        | 164        |
| Nkx6.2 does not seem to regulate the V2 population diversification                                                                                                 | 165        |
| Nkx6.2 controls the distribution of V2 interneurons                                                                                                                | 168        |
| 3.1.1. Discussion                                                                                                                                                  | 171        |
| <b>4. General discussion</b>                                                                                                                                       | <b>173</b> |
| 4.1. <i>The ventral IN diversification</i>                                                                                                                         | 174        |
| 4.2. <i>The OC factors control the V2a IN diversification</i>                                                                                                      | 177        |
| 4.3. <i>The OC factors control the V2 IN distribution</i>                                                                                                          | 179        |
| 4.4. <i>OC factors control the expression of Pou2f2 and Nkx6.2</i>                                                                                                 | 182        |
| 4.5. <i>OC-Pou2f2 and OC-Nkx6.2 genetic cascades control the V2 distribution</i>                                                                                   | 184        |
| <b>5. Bibliography</b>                                                                                                                                             | <b>187</b> |

## Foreword

The locomotion is a complex behavior, which requires tight control and fine coordination of the limb movements. Although the regulation is initiated in the brain, the limb rhythmic activity is generated, coordinated and finely regulated by neuronal circuits located all along the antero-posterior axis of the ventral region of the spinal cord. These neuronal circuits, named central pattern generators (CPGs), are able to generate autonomously a stereotyped activity characterized by a certain rhythm and speed, a left-right alternation, a flexor-extensor alternation and a fore and hindlimb alternation. They are composed of motor neurons (MNs) and of interneuron (IN) populations. Ventral IN populations are generated during the embryonic stage and derive from 4 distinct cardinal populations, named V0, V1, V2 and V3. Each ventral population migrates to a very precise position within the spinal cord to adequately integrate the locomotor circuits. It has been shown that each ventral population diversifies into multiple subpopulations, which play complementary functions during locomotion allowing the flexibility and the versatility of the locomotor CPGs (Gosgnach et al., 2017). Indeed, the V0 INs are divided in 2 major populations, namely the inhibitory V0<sub>D</sub> and the excitatory V0<sub>V</sub>, which control the left-right alternation during locomotion at low and high speed, respectively (Talpalar et al., 2013). *Satb2* dorsal inhibitory INs are divided into 2 subpopulations depending on their differential expression of *Ctip2* (Hilde et al., 2016). The V1 INs diversify into ~50 subpopulations based on the combined expression of transcription factors but also on their electrophysiological properties (Bikoff et al., 2016; Gabitto et al., 2016). The V2 are composed of 2 major populations, V2a and V2b, and 2 minor populations, V2c and V2d. The excitatory V2a are fractionated into 2 major types: type I V2a INs that connect directly to local MNs and type II V2a INs that display local and supraspinal

bifurcating connections. Moreover, the V2a population can be divided into additional subpopulations after birth (Bikoff et al., 2016; Hayashi et al., 2018). Finally, V3 INs are segregated into spatially and functionally distinct clusters along the dorso-ventral axis of the spinal cord, each showing distinctive maturation processes and likely playing different roles in motor activity (Borowska et al., 2015; Borowska et al., 2013). However, the mechanisms controlling the diversification of ventral populations into subpopulations and how each subpopulation is involved in the locomotor coordination are poorly understood.

In addition to the diversification, the distribution of each ventral population and subpopulation has been demonstrated as a key determinant of microcircuit organization. *Satb2* regulates the *Satb2*<sup>+</sup> neuron localization on the mediolateral axis of the spinal cord, which leads to a proper integration in motor circuits (Hilde et al., 2016). V1 subsets occupy highly structured spatial distributions that constrain patterns of input from sensory and motor neurons (Bikoff et al., 2016). In addition, their distinct distribution along the rostro-caudal axis differentiates limb-specific and thoracic-specific V1 INs suggesting differential motor output at thoracic or at lumbar level of the spinal cord (Sweeney et al., 2018). Similarly, V2a type I and II INs are distributed according to a counter-gradient along the antero-posterior axis of the spinal cord that drives unique patterns of motor activity at brachial and lumbar levels (Hayashi et al., 2018). Finally, dorsal and ventral V3 INs are functionally distinct: both dorsal and ventral V3 are recruited at running but only ventral V3 are recruited at swimming (Borowska et al., 2015; Borowska et al., 2013).

My thesis project sought to identify the molecular mechanisms that regulate proper distribution of spinal INs in order to adequately integrate neuronal circuits.

## **1. Introduction**

### **1.1. Spinal cord regionalization**

The central nervous system (CNS) derives from the neural plate that arises from the dorsal ectoderm. The neural plate gives rise to the forebrain, midbrain, hindbrain, and the neural tube. The spinal cord is composed of distinct neuronal populations. The neuronal populations located in the dorsal region of the dorso-ventral axis of the spinal cord relay sensory information from the periphery to the brain, while the ventral populations coordinate the motor outputs to the muscles. During neurogenesis, the production of neuronal populations in the developing spinal cord depends on signals, such as: Sonic Hedgehog (Shh), Bone Morphogen Proteins (BMPs), Wnt ligands, retinoic acid (RA) and Fibroblast Growth Factors (FGFs), that control the genetic networks leading to the dorso-ventral and antero-posterior patterning.

#### **1.1.1. Antero-posterior patterning of the developing spinal cord**

Several signals establish the antero-posterior regionalization of the neural plate. The most anterior part of the neural plate forms the forebrain, and the posterior parts progressively give rise to the midbrain, the hindbrain and the spinal cord. Therefore, the formation of the spinal cord needs posteriorizing signals, such as: FGF, RA, and some Wnt factors. Gradients of FGF, RA and Wnt induce the expression of Hox4 to Hox11 group genes, and determine their spatial expression in MN and IN progenitors along the antero-posterior axis of the neural tube. The cross-repression between Hox proteins in newborn neurons regionalizes the neural tube and defines 3 distinct parts: brachial, thoracic and lumbar regions.

Hence, Hox6 defines neuronal populations in the brachial region, Hox9 is essential for the generation of thoracic populations, and finally Hox10 is required for the lumbar populations (see the explanation below in the chapter dedicated to MNs) (Ciani and Salinas, 2005; Elkouby and Frank, 2010; Gaspard and Vanderhaeghen, 2010; Jung et al., 2010; Maden, 2002; Philippidou and Dasen, 2013; Sweeney et al., 2018).

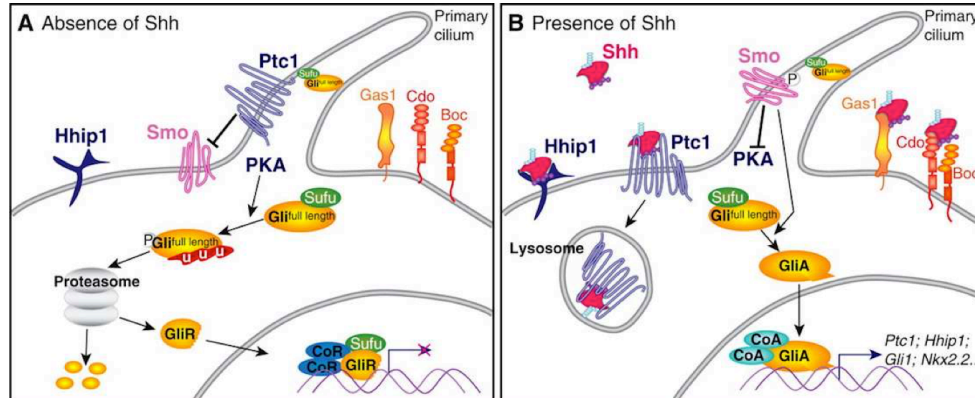
### **1.1.2. Dorsal-ventral patterning of the spinal cord**

Distinct neuronal populations are produced along the dorso-ventral axis under the action of morphogens. BMPs, belonging to the TGF $\beta$  superfamily, and Wnt factors are produced and secreted first by the ectoderm then by the roof plate. They form a signaling gradient in the dorsal part of the spinal cord required for the production of 6 dorsal progenitor domains that give rise to 8 dorsal IN populations (see below). In contrast, Shh is a crucial morphogen produced and secreted first by the notochord then by the floor plate that establish the ventral patterning in the neural tube. BMP and Shh signalings have antagonist activities and BMP acts by limiting the ventral neural tube development (Briscoe and Novitsch, 2008; Dessaud et al., 2008; Gammill and Bronner-Fraser, 2003; Goulding and Lamar, 2000).

### **1.1.3. Ventral patterning mediated by Shh**

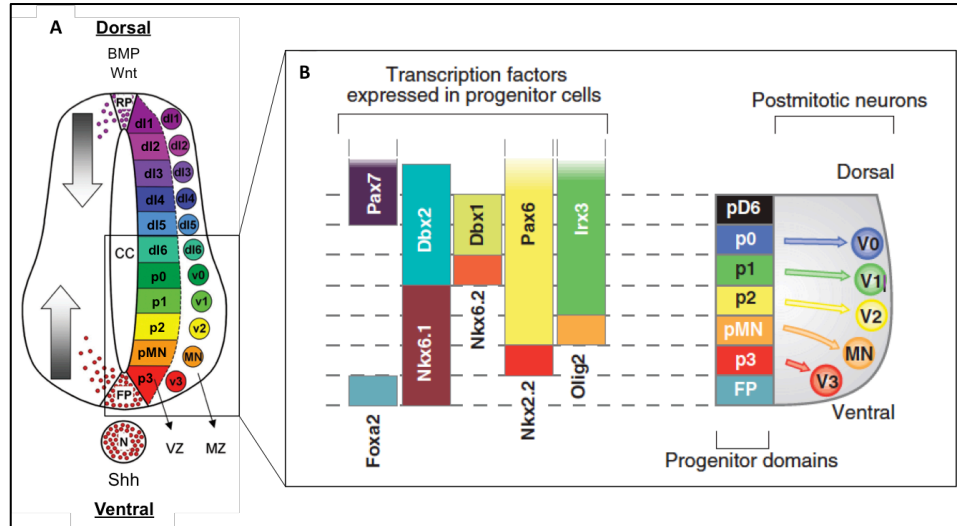
Shh is produced and secreted first by the notochord then by the floor plate. After its translation Shh undergoes auto-cleavage and lipid modifications. Active Shh (ShhNp) has a cholesterol and a palmitate group attached at the carboxy-terminal and the amino-terminal ends, respectively. Afterwards, ShhNp is released from the producing cell by a multi-pass transmembrane protein, named

dispatched 1 (Disp1), and multidimerizes. How Shh spreads in the neural tube remains elusive. Nevertheless, recent studies highlighted that several extracellular and transmembrane proteins, like Heparin sulphate proteoglycans, Ptch1, Hhip1, Gas1, Cdo and Boc, interact with Shh to decrease its diffusion through the ventral neural tube and either reduce or increase its signaling. By spreading within the neural tube, Shh form a ventro-dorsal signaling gradient that controls the production of distinct progenitor domains (Briscoe and Ericson, 1999; Dessaud et al., 2008; Jessell, 2000; Marti and Bovolenta, 2002). Shh binds to its receptor Patched1 (Ptc1) and allows the recruitment of a co-receptor Smoothened (Smo) to the membrane of the primary cilium to transduce the signal. In absence of Shh, Ptc1, located in the primary cilia, inhibits the Smo recruitment (Briscoe and Small, 2015; Dessaud et al., 2008). Smo activates the zing-finger transcription factor of the Gli family, key regulators of the Shh signaling (Figure 1). Gli family contains 3 members, Gli1, 2 and 3, which are characterized by five tandem C2H2 zinc-fingers that comprise the DNA-binding domain. All the 3 members contain a C-terminal activation domain, while only Gli2 and Gli3 contain a N-terminal repressor domain. Gli1 and Gli2 act as activators whereas Gli3 acts as a repressor. Activated Smo prevents the degradation of Gli activator to Gli repressor and promotes the activated Gli transport from the primary cilia to the nucleus to induce the expression of target genes (Figure1). The concentration gradient of Shh establishes a gradient of Gli activity by inhibiting Gli repressor activity and stimulating Gli activator activity. The duration as well as the concentration of Shh signaling influences the pattern of ventral progenitors (Briscoe and Ericson, 1999; Chamberlain et al., 2008; Dessaud et al., 2010; Jacob and Briscoe, 2003; Lee and Pfaff, 2001).



**Figure 1: Shh signaling transduction** (<http://cshperspectives.cshlp.org/content/1/2/a002014/F3.expansion.html>). **(A)** In the absence of Shh, Ptc1, located in the primary cilium, prevents the recruitment of Smo into the primary cilium. The Gli activator (GliA) is degraded to Gli repressor (GliR), which inhibits the target gene transcription. **(B)** When Shh binds to its receptor Ptc1, this last one move from the primary cilium allowing the Smo recruitment to the primary cilium. Smo prevent the GliA degradation in GliR allowing to GliA to stimulate the target gene transcription.

Shh signaling induces the expression of class II homeodomain (HD) transcription factors: Nkx6.1, Nkx6.2, Nkx2.2, Olig2, and represses the expression of class I HD transcription factors: Pax7, Dbx2, Dbx1, Pax6 and Irx3. A unique combination of transcription factors is determined by the Shh concentration gradient. Longer exposure to high Shh concentration is necessary to generate the most ventral progenitor domains whereas a shorter exposure to low Shh concentration is sufficient for dorsal progenitor domains. Furthermore, the mutual cross-repression between class I and II HD transcription factors delineates distinct ventral progenitor domain boundaries (Dessaud et al., 2008). Hence, five progenitor domains (p) are generated in the ventral neural tube: p0, p1, p2, pMN and p3 constituted by proliferative progenitors. These progenitor domains give rise to 1 class of MNs and to 4 classes of ventral INs: V0, V1, V2 and V3 that contribute to the locomotor control (Figure 2). In addition to the ventral IN populations, some dorsal INs, the dl3 and the dl6 INs, also participate in the control of locomotor coordination.



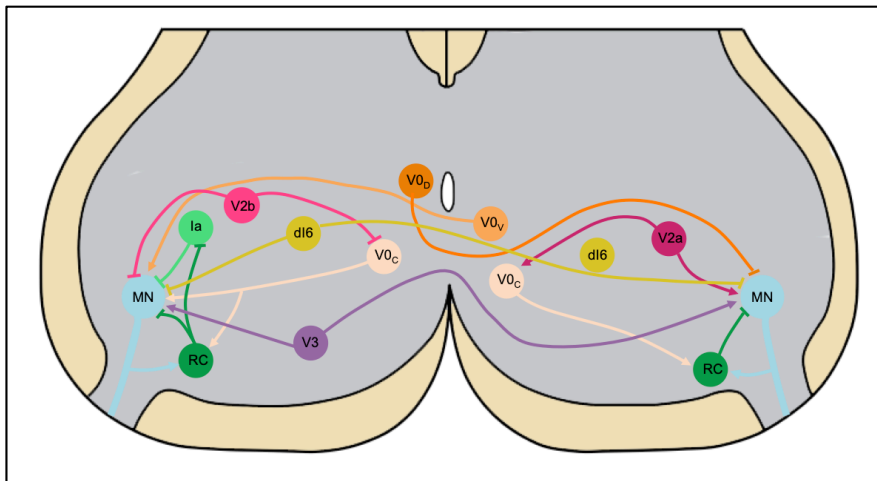
**Figure 2: Shh and BMP/Wnt signaling gradients induce the production of ventral and dorsal progenitor domains (adapted from (Dessaud et al., 2008; Ulloa and Briscoe, 2007)). (A)** Shh produced by the notochord (N) then by the floor plate (FP), induces the production of 5 ventral progenitor domains (p0-p3). In contrast, BMP and Wnt produced by the ectoderm then by the roof plate (RP), induce the production of 6 dorsal progenitor domains (dl1-dl6). These progenitor domains give rise to different neuronal populations. CC = center canal, VZ = ventricular zone, MZ = mantle zone. **(B)** Shh induces the expression of class II HD transcription factors: Nkx6.1, Nkx6.2, Nkx2.2, Olig2, and represses the expression of class I HD transcription factors: Pax7, Dbx2, Dbx1, Pax6 and Irx3. Shh concentration gradient determines a unique combination of transcription factors expressed by each progenitor domain. The mutual cross-repression between these class I and II HD transcription factors establishes well defined boundaries between progenitor domains.

## 1.2. Ventral neuronal circuits

As mentioned above, 5 neuronal populations are generated in the ventral region of the spinal cord. The MNs directly contact the muscles and the sympathetic chain in the periphery, while the ventral INs modulate MN activity by either directly innervating them or by contacting intermediate IN populations. The ventral INs follow different migratory routes, settle in distinct locations in the ventral spinal cord, project their axons differently and control distinct aspects of locomotion. Furthermore, the ventral INs can be subdivided into 2 types: commissural or non-commissural. Therefore, the V0 and the V3 populations



comprise commissural INs that innervate their targets by projecting their axons to the contralateral side of the spinal cord. In contrast, the V1 and the V2 INs, being not commissural, project their axon ipsilaterally to innervate their targets on the same side as their cell body. These ventral populations form with MNs complex neuronal circuits that control locomotion outputs (Figure3). The specification and the role of each ventral IN population in the control of the locomotor coordination have been extensively investigated and will be detailed below after a brief introduction to MNs.



**Figure 3: Schematic representation of neuronal circuits located in the ventral region of the spinal cord (Gosgnach et al., 2017; Ramirez-Jarquin and Tapia, 2018).** The CPGs are composed of MNs, ventral or dorsal IN populations. MNs directly innervate individual muscles, while INs modulate MN activity either by directly contacting them, or by innervating intermediate IN populations. The IN populations stimulate or inhibit their targets by projecting their axons ipsilaterally or contralaterally to their cell body.

### 1.2.1. Motor neurons

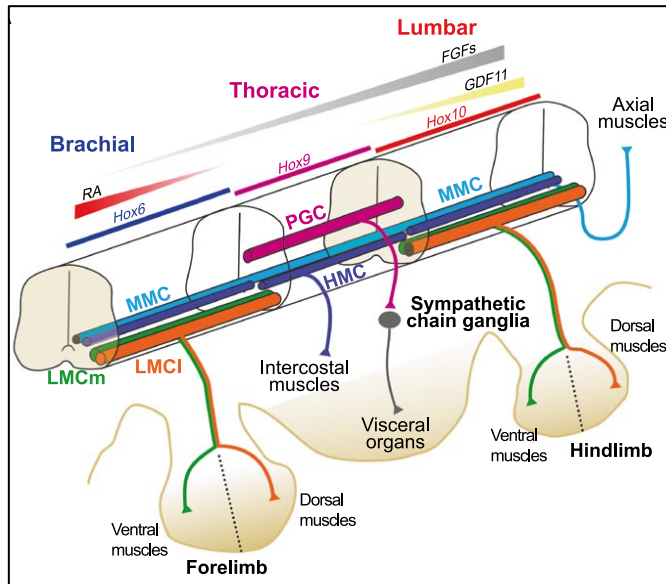
MNs derive from the pMN domain, which expresses a combination of class I and II HD and bHLH transcription factors: *Olig2*, *Nkx6.1* and *Pax6* that define the identity of MN progenitors (Figure 2). In addition to Shh, RA defines and

consolidates the pMN domain by regulating the expression of *Olig2*, *Irx3* and *Pax6*, independently from Shh. Then, different proteins, including Neurog2, Lhx3, Lhx4, Foxp2 and Foxp4 allowing cell cycle exit, promote MN differentiation. Once postmitotic, MN identity is consolidated by the expression of multiple transcription factors, including *Isl1* and *Hb9*, which mutually stimulate their expression in a positive feedback loop. Although distinct, MN and V2a populations are produced from neighboring progenitor domains and share the expression of *Lhx3* and *NLI*, forming a hexamer complex with *Isl1* in MNs and a tetramer complex in V2a INs. The hexamer complex in MNs stimulates the expression of *Hb9* that, in turn, on the one hand promotes MN differentiation and on the other hand inhibits the V2a consolidation program. It has been demonstrated that the inactivation of *Isl1* or *Hb9* promotes the V2a cell fate at the expense of MN production. The molecular mechanism is described below in the V2 interneurons chapter (see below) (Dasen and Jessell, 2009a; Francius and Clotman, 2014).

MNs are all cholinergic neurons. They display distinct molecular identities, localization, axonal trajectories and target connectivity. Indeed, they undertake different waves of diversification. First of all, MNs are segregated into visceral or somatic MNs. The visceral MNs are present only in the thoracic region, while the somatic MNs are located all along the antero-posterior axis of the spinal cord. Indeed, MNs are anatomically organized in columns and settle in a very defined position along antero-posterior and dorso-ventral axes of the spinal cord, regulated by Foxp1 and the Hox gene family. A single motor column located at thoracic level, the preganglionic column (PGC), gathers the visceral MNs that innervate the sympathetic ganglia. Three motor columns contain the somatic MNs. At thoracic level, the hypaxial motor column (HMC) innervates intercostal and abdominal wall musculature. At limb level, the lateral motor column (LMC)

innervates limb muscles. This column is further subdivided into 2 subsets: the medial division of the LMC (LMCm) with axons projecting ventrally through the limb mesenchyme and the lateral division of the LMC (LMCl) that innervates the dorsally located limb muscles. Finally, the median motor column (MMC) present all along the antero-posterior axis projects axons towards the dorsal axial musculature (Figure 4) (Dasen and Jessell, 2009a; Francius and Clotman, 2014).

As mentioned above, *Hox* genes establish the regionalization of motor columns along the antero-posterior axis of the spinal cord. Early in MN progenitors, the *Hox* gene expression is regulated by multiple mesodermal signaling gradient of morphogens, namely: FGF, RA and Gdf11 (Figure 4). FGF signaling gradient establishes the initial induction of *Hox* gene expression at each level of the spinal cord, so that the more caudal regions receive the higher FGF concentration and express 5' *Hox* clusters. RA signaling present at more rostral regions regulates *Hox* expression at brachial region of the spinal cord. Finally, Gdf11, a member of the TGF $\beta$  superfamily, expressed at more caudal regions of the spinal cord controls *Hox* gene expression at thoracic and lumbar regions. It is the cross-repression of specific *Hox* proteins that defines distinct MN identities and regionalizes the motor columns along the antero-posterior axis of the spinal cord. Hence, *Hox6* is expressed by brachial MNs, *Hox9* by thoracic MNs and *Hox10* by lumbar MNs (Figure 4). Several studies have shown that modulating the expression of those 3 groups of paralog *Hox* genes results in a modification of the motor column organization. For instance, absence of *Hoxc9*, normally expressed by thoracic MNs, in a mouse model makes the thoracic motor columns acquire a brachial LMC identity (Dasen and Jessell, 2009a; Francius and Clotman, 2014; Jung et al., 2010; Philippidou and Dasen, 2013).



**Figure 4: Topographic organization of motor columns along the antero-posterior axis of the spinal cord (Francius and Clotman, 2014).** FGFs, RA and GDF11 gradients establish spatial expression of Hox genes along the antero-posterior axis of the spinal cord. In turn, Hox genes induce the regionalization of the spinal cord and define 3 regions: brachial, thoracic and lumbar levels. Hox6 group defines the brachial level, Hox9 the thoracic level and Hox10 the lumbar level. At limb level,

MNs are organized in 2 motor columns: the LMCI and LMCm, which innervate the dorsal and the ventral limb parenchyma, respectively. At thoracic level, HMC neurons innervate the intercostal muscle, while the PGC neurons innervate the sympathetic chain ganglia. Finally, MMC neurons present all along the spinal cord, innervate the dorsal axial musculature.

In addition to their division in 2 columns, the LMC neurons further subdivide in more than 50 motor pools in order to innervate individual muscles. As for the motor columns, the combinatorial expression of *Hox* genes along the antero-posterior axis of the LMCs is also required for the motor pool diversification and regionalization. Many other proteins, downstream of Hox factors or activated by the GDNF signaling, are involved in the specification of motor pools, as Nkx6.1 and PEA3 (Dasen and Jessell, 2009a; Francius and Clotman, 2014).

### 1.2.2. Commissural interneurons

V0 and V3 INs are gathered in this chapter based on their predominantly axonal projection to the contralateral side of the spinal cord. The commissural INs ensure the left-right coordination of MN activity.

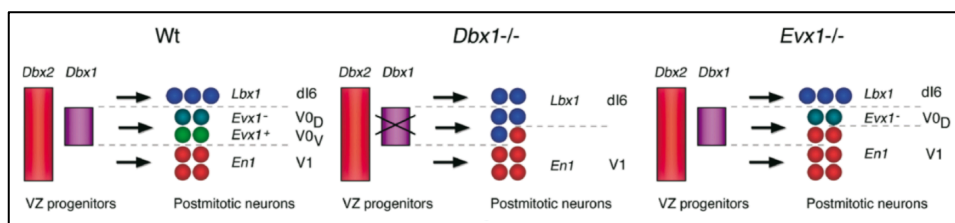
### V0 interneurons

The V0 INs emerge in the intermediate area from the p0 domain, which is located in the most dorsal part of the ventral half of the neural tube. This domain, which expresses the HD transcription factors *Dbx1*, *Dbx2*, *Irx3* and *Pax6*, requires the weakest Shh signal to promote its identity. Shh signaling appears to be sufficient but not necessary for the V0 differentiation. Indeed, RA can induce, like Shh but through an independent pathway, the generation of p0 progenitors. However, *in vitro* studies demonstrated that RA is not required for the generation of the p0 progenitors but for the V0 IN production once the progenitors are established (Pierani et al., 1999).

The V0 cardinal population consists of 4 populations: dorsal V0 (V0<sub>D</sub>), ventral V0 (V0<sub>V</sub>) characterized by the expression of *Evx1*, and 2 more discrete populations derived from the V0<sub>V</sub> INs, cholinergic V0 (V0<sub>C</sub>) and glutamatergic V0 (V0<sub>G</sub>) both characterized by *Pitx2* expression (Griener et al., 2015; Moran-Rivard et al., 2001; Zagoraïou et al., 2009). These populations are distinct from each other by their molecular identity, migratory route, final localization, axonal trajectory and neurotransmitter. The V0<sub>D</sub> and V0<sub>V</sub> INs are commissural neurons and project their axons towards the contralateral side of the spinal cord. The V0<sub>D</sub> INs, which represent two-thirds of the entire V0 population, are GABA- and glycinergic and control the left-right alternation at low speed during locomotion by inhibiting MN activity. The V0<sub>V</sub> INs are glutamatergic and stimulate the contralateral MNs activity. With the V2a population that innervates them, the V0<sub>V</sub> are necessary for the hindlimb left-right alternation at high speed. Studies have shown that the specific deletion of V0<sub>D</sub> or V0<sub>V</sub> in mouse models leads to a synchronization of left and right limbs depending on the speed locomotion. These populations also receive inputs from sensory afferents or other interneuron populations, capable to modulate their activity. Hence, studies have shown that both V0<sub>D</sub> and V0<sub>V</sub> INs

receive serotonergic inputs, while only the  $VO_D$  INs are contacted by primary sensory afferent inputs (Crone et al., 2008; Crone et al., 2009; Griener et al., 2015; Lanuza et al., 2004). Finally, the  $VO_C$  and  $VO_G$  INs are ipsilaterally projecting neurons. Although the role of the  $VO_G$  INs is yet unknown, the cholinergic  $VO_C$  INs are able to modulate the task-dependent locomotor behavior (switch from walking to swimming for instance) by being the sole source of C bouton inputs to MNs (Lanuza et al., 2004; Talpalar et al., 2013; Zagoraoui et al., 2009). This population also innervates the V1-derived Ia INs (Siembab et al., 2010).

*Dbx1* is a key transcription factor sufficient and necessary for the differentiation of all the  $VO$  populations. Indeed, *Dbx1* regulates the expression of *Evx1* that in turn is required for  $VO_V$  differentiation and migration. In *Dbx1* mutant mice, cells exhibiting characteristics of dl6 or V1 INs are produced at the expense of all  $VO$  INs. In the *Evx1* mutant mice, only the  $VO_V$  are affected and replaced by V1 INs (Figure 5). Finally, *Dbx1* expression is dorsally repressed by *Pax3* and *Pax7* present in dorsal progenitors and ventrally repressed by *Prdm12* present in the p1 progenitor domain (Gard et al., 2017; Moran-Rivard et al., 2001; Pierani et al., 2001; Thelie et al., 2015).



**Figure 5:  $VO$  IN production in *Dbx1* and *Evx1* mutant mice (Vallstedt et al., 2001).** In wild-type (Wt) mice, *Dbx1*<sup>+</sup> progenitors give rise to *Evx1*<sup>-</sup>  $VO_D$  and *Evx1*<sup>+</sup>  $VO_V$  INs. In *Dbx1*<sup>-/-</sup> mutant mice, dl6 and V1 INs are produced at the expense of all  $VO$  INs. Finally, in *Evx1*<sup>-/-</sup> mutant mice, V1 INs are produced at the expense of  $VO_V$  INs, while  $VO_D$  INs are unaffected.

### V3 interneurons

The V3 INs are produced from the most ventral progenitor domain, p3. The p3 domain is characterized by the combinatorial expression of *Nkx6.1*, *Nkx2.2*, and *Nkx2.9*. Several studies demonstrated that *Nkx2.2* and *Nkx2.9* play redundant role in establishing the p3 progenitor domain and ensure together its identity. However, *Nkx2.2* alone is capable to regulate the production of V3 INs. Indeed, MMC neurons are produced at the expense of V3 INs in *Nkx2.2*<sup>-/-</sup> mutant mice, while the p3 progenitor domain remains intact. In contrast, both p3 domain and V3 INs are affected and replaced by Olig2+ MN progenitors and MMC neurons in *Nkx2.2*<sup>-/-</sup>;*Nkx2.9*<sup>-/-</sup> compound mutant mice (Briscoe et al., 1999; Holz et al., 2010; Jarrar et al., 2015).

The V3 cardinal population characterized by *Sim1* expression consists of 2 distinct populations based on their distribution: the dorsal V3 (V3<sub>D</sub>) and the ventral V3 (V3<sub>V</sub>) INs. The vast majority of V3 INs are commissural neurons and projects their axons toward the other half of the spinal cord, while a subset of V3 (15%) are ipsilateral projecting neurons. The V3 INs project their axons onto the MNs and V1 INs and hence ensure a proper walking gait by promoting a robust, organized and balanced locomotor activation on both sides of the spinal cord. Indeed, they coordinate rhythmic motor outputs from each half of the spinal cord. The involvement of each V3 population in the control of locomotor activity is currently unknown. However, it has been demonstrated using a mouse model that both V3<sub>D</sub> and V3<sub>V</sub> INs are actively recruited during running, while only V3<sub>V</sub> INs are preferentially activated during swimming. Also, although all glutamatergic, these 2 populations display distinct electrophysiological, morphological and structural properties. The V3<sub>V</sub> INs are also molecularly distinct from the V3<sub>D</sub> INs by the expression of *Olig3* induced by *Nkx2.2* (Borowska et al., 2013; Liu et al., 2014; Zhang et al., 2008).

The V3 INs migrate dorsally and laterally from their production site and settle along the dorso-ventral axis of the spinal cord within 3 clusters at e14.5 in mice: dorsal, intermediate and ventral clusters. The intermediate cluster is a combination of both V3<sub>D</sub> and V3<sub>V</sub> INs. *Sim1*, a PAS-bHLH transcription factor, plays an essential role in the regulation of their migration and their commissural axon guidance. Indeed, despite the fact that the number of V3 INs is normal in *Sim1* mutant mice, they are abnormally distributed with a decrease of the dorsal cluster and an increase of the intermediate cluster. Furthermore, one observes a decrease of the contralateral projecting V3 INs without affecting the ipsilateral projections, suggesting that the loss of *Sim1* does not prevent the axons to cross the midline but affects the longitudinal growth of axons once they have crossed the floor plate (Blacklaws et al., 2015; Zhang et al., 2008).

### 1.2.3. Non commissural interneurons

V1 and V2 INs are gathered in this chapter based on their axonal projection in the ipsilateral side of the spinal cord.

#### V1 interneurons

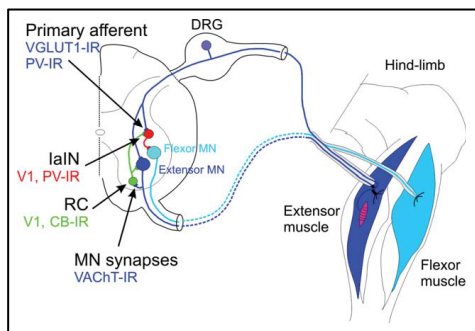
V1 INs characterized by the expression of *Engrailed 1 (En1)*, arise from the p1 progenitor domain expressing *Pax6*, *Dbx2* and *Nkx6.2*, which define the p1 identity (Figure 2). The p1 domain, located just ventral to the p0 domain, also requires a weak Shh concentration for its production. Therefore, Shh and RA act in parallel for p1 progenitor and V1 IN production, as they do for p0 and V0 INs. Many transcription factors contribute to the consolidation of the V1 cell fate. *Pax6* is required for the V1 IN production by controlling the expression of *Dbx2* and *En1* in the progenitors and in the differentiated V1 INs, respectively. *Prdm12*



consolidates the V1 cell fate by repressing dorsally *Dbx1* and ventrally *Nkx6.2* (Burrill et al., 1997; Ericson et al., 1997; Gosgnach et al., 2006; Matise and Joyner, 1997; Pierani et al., 1999; Sapir et al., 2004; Saueressig et al., 1999; Thelie et al., 2015; Vallstedt et al., 2001; Zannino et al., 2014).

After their differentiation, the V1 populations extend their axons ipsilaterally and rostrally via the ventrolateral funiculus (VLF) to specifically innervate somatic MNs. Pax6 regulates the early cell fate acquisition of V1 INs while *En1* controls their axon pathfinding. Indeed, in *En1* mutant mice, the V1 axons fasciculate in the VLF and fail to progress properly towards MNs (Betley et al., 2009; Sapir et al., 2004; Saueressig et al., 1999).

The V1 INs are subdivided in several GABA- and glycinergic populations: Ia INs, Renshaw cells (RCs), and other unknown V1, and display distinct localization, functional properties and synaptic inputs. The Ia inhibitory INs settle dorsally to the MN area, receive inputs from Ia muscle afferents and provide reciprocal inhibition to antagonistic motor pools (Figure 6) (Eccles et al., 1956). The RCs settle in a characteristic position between MNs and the ventral white matter border, receive excitatory inputs from MN axon collaterals and in turn mediate recurrent inhibition of MNs. They also modulate the activity of Ia INs (Figure 6). Finally, other V1 INs are part of the V1 cardinal population but have not yet been characterized (Alvarez et al., 2005; Eccles et al., 1954; Hultborn et al., 1971; Renshaw, 1946; Sapir et al., 2004; Siembab et al., 2010).



**Figure 6: Connectivity of Ia INs and Renshaw cells in adult mouse (Siembab et al., 2010).** The Ia INs are located dorsally to the MNs and project onto the flexor MN. They receive primary afferents from Ia sensory neurons located in the dorsal root ganglia (DRG). In contrast, RCs are located ventro-medially to the MNs. They receive inputs from MN axon collaterals and in turn project to the extensor MNs

The contribution of each V1 population to the control of the locomotion has not yet been investigated. However, the V1 INs control the locomotor rhythm speed. Indeed, mutant mice for *En1* fail to perform quick stepping movement, resulting in a slowed locomotor rhythm as compared to the control littermates (Gosgnach et al., 2006). In addition, the V1 control together with the V2b INs the flexor-extensor alternation of antagonistic muscles (Zhang et al., 2014)

### V2 interneurons

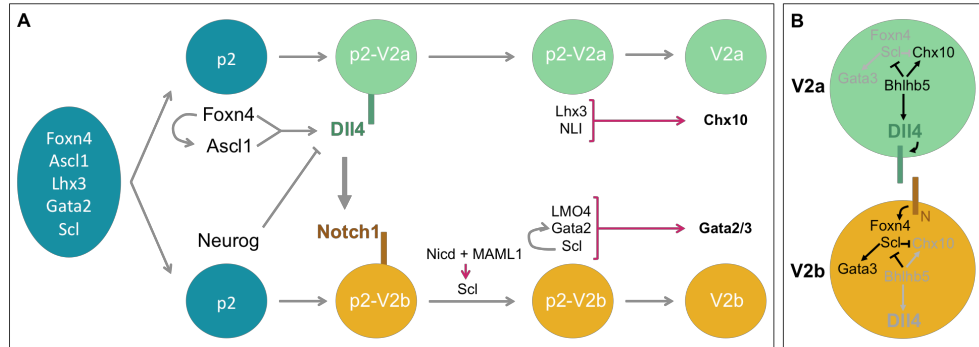
The p2 progenitor domain gives rise to 2 main populations, the V2a and V2b INs and 2 minor populations, the V2c and V2d INs, which derived from the V2b and V2a INs. These progenitors express HD transcription factors *Pax6*, *Irx3*, and *Nkx6.1*, that define the p2 identity. Other transcription factors and signaling pathways are responsible for the specification of V2a and V2b from a common p2 progenitor domain.

### Cell fate specification and diversification

Foxn4, *Ascl1*, *Lhx3* and *Gata2* constitute the early transcription factor code in the p2 progenitors in combination with the above-mentioned HD factors (Del Barrio et al., 2007; Peng et al., 2007). Foxn4, a winged/forkhead transcription factor, expressed in all the progenitors, is a key determinant of V2b cell specification by setting and maintaining the mosaic expression of *Ascl1* and *Neurogenin1/2* proneural genes in the p2 progenitors. Indeed, Foxn4 induces the expression of *Acs1* in some precursors, which in turn forms a heterodimer with E12 to induce the expression of *Delta-like4* (*Dll4*), a Notch signaling ligand. Both Foxn4 and *Ascl1* synergistically stimulate *Dll4* expression by binding to the CR1 enhancer. Although Neurogenin factors also form heterodimers with E12 and bind

to the CR1 enhancer, they are unable to activate *Dll4* expression. This results in an asymmetric *Dll4* expression in V2 precursors (Misra et al., 2014). *Dll4* expressed by some V2 precursors triggers the Notch signaling in neighboring cells by binding to its receptor Notch1, whose expression oscillates in the V2 precursors. The Notch signaling activation leads to the cleavage of Notch intracellular domain (Ncd) of Notch receptor. Once transferred to the nucleus, Ncd through association with MAML1, activates *Gata2* and *Scf* expression. Hence, Ncd activates the *Scf*-dependent V2b IN fate program. It has been shown that *in ovo* electroporation of a *Scf* expression vector in chicken embryos is sufficient to compensate for the loss of the Notch signaling to induce the V2b IN specification (Peng et al., 2007). Thus, the *Dll4*-Notch signaling promotes the generation of distinct V2 precursor subsets, the V2a and V2b precursors (p2-V2a and p2-V2b). Therefore, the precursor cells wherein Notch signaling is activated are V2b precursors, while the precursors expressing *Dll4*, in which Notch signaling remains inactive, are V2a precursors (Figure 7A). The Notch/delta signaling also controls the specification of V2a and V2b IN fates in zebrafish (Batista et al., 2008; Kimura et al., 2008).

Furthermore, the BMP-TGF $\beta$  signaling pathway is involved, downstream of the Notch signaling, in promoting the V2b cell fate. Indeed, in *Smad3* and *Smad4* (2 BMP/TGF $\beta$  signal transducers) compound mutant mice, the number of V2b INs is reduced while the number of V2a INs is unaffected (Misra et al., 2014). Finally, *Bhlhb5* appears to play 2 important roles in the V2 specification. On the one hand, *Bhlhb5* promotes in a cell autonomous way the formation of V2a INs in which it is expressed. On the other hand, *Bhlhb5* promotes non-cell autonomously the V2b cell fate by directing the *Dll4* expression in p2-V2a allowing to trigger the Notch signaling in p2-V2b (Figure 7B) (Skaggs et al., 2011).



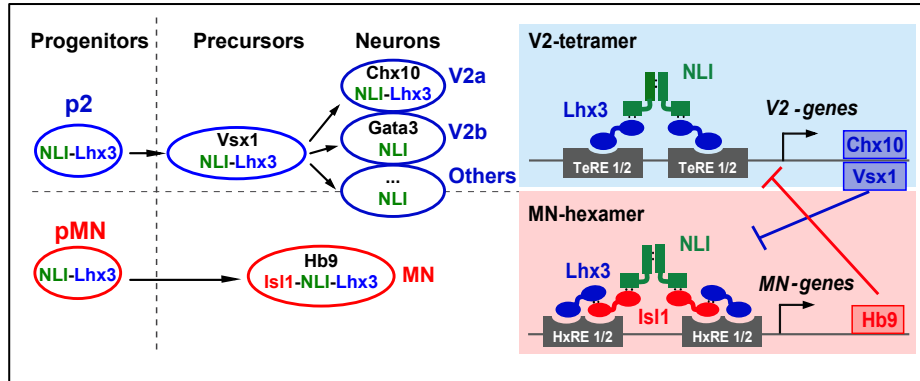
**Figure 7: V2 cell fate specification and diversification (Peng et al., 2007; Skaggs et al., 2011).** (A-B) Under the action of specific transcription factor networks, V2a and V2b populations are produced from a unique p2 progenitor domain. See details in the text. N = Notch1

V2c and V2d INs derive from the V2b and V2a populations, respectively. However, their specification mechanism has been less investigated.

### Cell fate consolidation

The V2 INs are produced from the p2 progenitor domain that is directly dorsal to the pMN progenitor domain and shares the expression of Lhx3 and NLI. Studies have shown that these 2 proteins are part of distinct complexes in postmitotic cells, involved in the consolidation of V2a and MN identities. In V2a INs, NLI and Lhx3 form a tetramer complex, in which the N-terminus of NLI self-dimerizes and its C-terminus interacts with the LIM domain of Lhx3. This V2-tetramer (2NLI:2Lhx3) binds to a DNA sequence named tetramer response element (TeRE) and regulates the V2a specific gene expression, including *Chx10* (Figure 8) (Lee et al., 2008; Thaler et al., 2002). In MNs, Lhx3 and NLI are part of another complex, the MN-hexamer complex, composed of NLI, Lhx3 and Isl1 in early-born MNs. In this complex, Isl1 binds simultaneously NLI by its LIM domain at its N-terminus and the LIM domain of Lhx3 by its C-terminus. The MN-hexamer (2NLI:2Lhx3:2Isl1) is responsible for the MN identity by binding a hexamer response element (HxRE) allowing the specific expression of MN genes, including

*Hb9* (Figure 8) (Lee et al., 2008; Thaler et al., 2002). The segregation of these 2 populations is ensured by the reciprocal feedforward control in the V2 INs and the MNs. Indeed, the V2a INs specifically express *Chx10*, a paired HD transcription factor with a CVC domain, which is critical for their development. Chx10 exerts a dual regulatory role to consolidate the V2a cell identity. On the one hand, Chx10 promotes V2a fate specification by transcriptional activation of specific V2a genes. On the other hand, it represses MN gene expression by binding the HxRE, hence, preventing uncharacterized complexes to bind the HxRE and to stimulate the MN gene expression (Figure 8) (Clovis et al., 2016). Moreover, we did show in our laboratory that *Vsx1*, a *Chx10* paralog, ensures the V2 identity consolidation at earlier stages of differentiation. Indeed, in V2 precursors, *Vsx1* is able to bind to the HxRE to repress MN gene expression in order to consolidate the V2 identity. In simple mutant mouse embryos for *Chx10* or *Vsx1*, no upregulation of *Hb9* expression is observed. However, double mutant for *Chx10* and *Vsx1* display an upregulation of *Hb9* expression, demonstrating partial redundancy of Chx10 and *Vsx1*. These findings also confirm that other complexes than MN-hexamer are able to bind the HeRE to induce the MN gene expression in the absence of the V2 identity consolidation program (Debrulle et al., in preparation). Oppositely, *Hb9*, a MN-specific HD transcription factor, binds to the TeRE and inhibits V2a gene expression to consolidate the MN identity (Figure 8) (Clovis et al., 2016; Lee et al., 2008). In the *Hb9* mutant mice, MNs are reduced by 58%, while Chx10 is upregulated in the MN area and is present in MN/V2 hybrid cells. In contrast, *Hb9/Chx10* compound mutant mice display 28% reduction of MNs and no change in the number of V2a INs, indicating that the MN deficit in *Hb9* mutant mice is in part caused by *Chx10* upregulation in the MN area (Clovis et al., 2016).

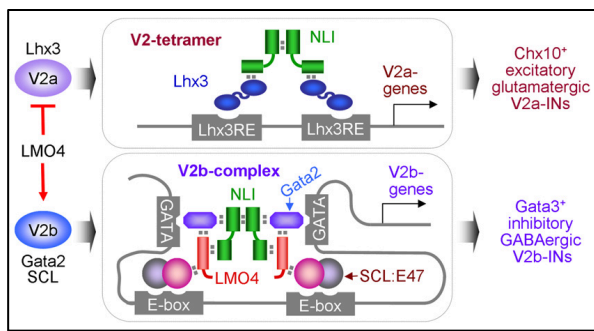


**Figure 8: Consolidation of the V2 and MN identities in precursors and differentiating cells (Stephanie Debrulle, submitted).** V2 INs and MNs are produced from adjacent progenitor domains that share *NLI* and *Lhx3* expression. Proteic complexes, V2-tetramer and MN-hexamer, consolidate the V2 and MN identities. The V2-tetramer (2NLI:2Lhx3) binds to the TeRE and regulates the V2a specific gene expression, including *Chx10*. In early V2 precursors, *Vsx1* ensure the consolidation of the V2 identity, while in differentiating V2a neurons, *Chx10* ensure their identity. The MM-hexamer (2NLI:2Lhx3:2Isl1) binds to the HxRE and stimulates MN gene expression, including *Hb9*. The segregation of V2 and MN populations is ensured by the reciprocal feedforward control from *Chx10* and *Vsx1* in V2 INs and *Hb9* in MNs.

In addition, the single-stranded DNA-binding proteins 1 and 2 (*Ssdp*), functionally redundant, are transcriptional coactivators of V2-tetramer and MN-hexamer by direct interaction between their N-terminal portion and the *NLI* protein. Indeed, in the *Ssdp1/Ssdp2* compound mutant mice, the number of specified V2a INs and MNs is reduced during spinal cord development. Furthermore, in these mutants, the H3-acetylation levels of V2a and MN genes are significantly reduced, suggesting that *Ssdp1* and *Ssdp2* mediate the recruitment of histone modifying complexes to induce histone H3-acetylation and histone H3-lysine4 trimethylation (H3K4me3) on V2-tetramer and MN-hexamer targets (Lee et al, 2016).

On the other hand, the V2b specification is consolidated by a V2b-complex composed of *NLI*, *Gata2*, *LMO4* and *Scl*. This complex directs the V2b gene expression by binding an enhancer composed of a pair of E-box and GATA composite elements found in the regulatory regions of *Gata2* and *Gata3* genes

(Figure 9). It appears that LMO4, a LIM-HD protein that lacks the functional DNA-binding domain, is likely essential for the assembly and the stabilization of the V2b-complex. Indeed, in *LMO4* null mice, the V2b-complex recruitment on the *Gata2/3* enhancer is severely attenuated with an increase in the number of Chx10+ V2a IN. Thus, LMO4 plays an important role in maintaining the balance between V2a and V2b INs (Figure 9) (Joshi et al., 2009; Lee et al., 2008).



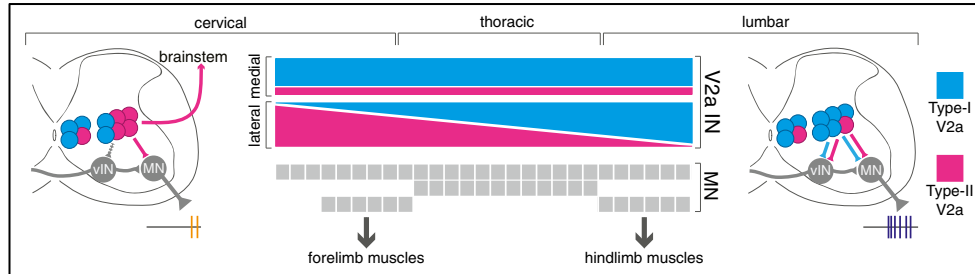
**Figure 9: The V2a and V2b identity consolidation (Joshi et al., 2009).** V2a identity is consolidated by V2-tetramer complex (2NLI:2Lhx3), which binds to a pair of tetramer response elements (Lhx3RE=TeRE) to induce V2a gene expression. The V2b identity is consolidated by a V2b-complex (2NLI:2Gata2:2LMO4:2SCL), which binds to a pair of E-box/GATA composite elements to induce V2b gene expression.

### The V2 populations

The V2a INs are ipsilateral excitatory neurons. They are subdivided in 2 types distributed in counter gradients along the antero-posterior axis of the spinal cord. The type I V2a INs, which maintain *Chx10* expression, are present all along the antero-posterior axis of the spinal cord but are enriched in the lumbar region. They are premotor INs and directly connect to ipsilateral MNs but also indirectly to contralateral MNs via commissural VO<sub>v</sub> INs (figure 10). The type I V2a INs control the left-right alternation during locomotion at high speeds. Studies of locomotor activities on Chx10-DTA mice, in which the V2a population is ablated, demonstrated that these mice progressively switch from an alternating gait to a hopping gait as the locomotion speed increase. Furthermore, in this mutant, the variability in burst amplitude and cycle period is more important indicating that

the V2a population could also stabilize the locomotor rhythm (Crone et al., 2008; Crone et al., 2009; Hayashi et al., 2018; Talpalar et al., 2013). In contrast, type II V2a INs progressively (from e12.5 in mouse) downregulate *Chx10* expression and are more numerous in the cervical spinal cord. In this region, they are propriospinal INs with bifurcating projections towards supraspinal neurons, likely the cerebellum via the lateral reticular nucleus, and local projections contacting ipsilateral or contralateral forelimb MNs (Figure 10). The selective ablation of these neurons perturbs the forelimb target reaching phase behaviour (Azim et al., 2014; Hayashi et al., 2018). Hence, these type I and II V2a subsets drive distinct motor activity patterns at brachial and lumbar levels. Finally, the rhombencephalic V2a neurons, within the CPGs located in the hindbrain, play an important role to maintain regular breathing in newborn mammals (Crone et al., 2012). At neonatal stage, V2a can be divided into 3 major subpopulations based on their intrinsic firing properties. In response to depolarizing current step, V2 INs fire tonically, phasically or with a delay. Tonic V2a INs display a tonic depolarization throughout the current step. Phasic V2a INs fire at the beginning of the depolarizing step then stop. Finally, the last V2a group fire tonically after a delay at the beginning of the depolarizing current step (Zhong et al., 2010). Moreover, type I and II V2a subsets can be further subdivided in 11 subpopulations based on the combinatorial expression of transcription factors (Hayashi et al., 2018).



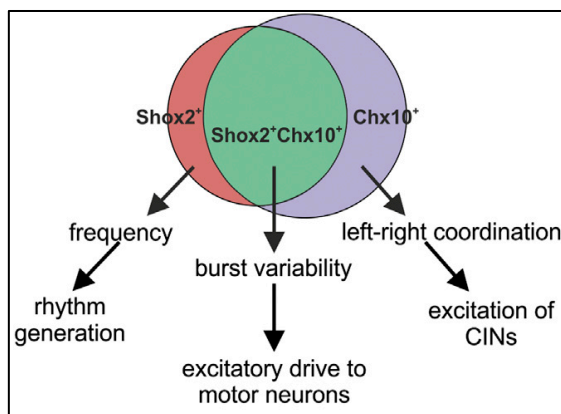


**Figure 10: Topographic distribution of the type I and II V2a interneurons (Hayashi et al., 2018).** The type I and II are distributed in 2 groups on the medio-lateral axis of the spinal cord. The V2a lateral group composed of type I and II INS, are distributed in counter-gradients along the antero-posterior axis of the spinal cord that drive unique patterns of motor activity at brachial and lumbar levels. The type I V2a INs, enriched in the lumbar region, contact the local MNs. In contrast, type II V2a INs, more numerous in the cervical spinal cord, display local and supraspinal bifurcating connections. The medial group of V2a mostly composed of type I V2a INs axial premotors.

V2b INs are ipsilaterally projecting inhibitory neurons. They directly innervate MNs and V0<sub>c</sub> INs. In addition, like the V1 INs, a subpopulation of V2b INs has characteristics of Ia INs and receive high-density convergent inputs from RC axons and vGluT1+ primary sensory afferents. Together with the V1 INs, the V2b population controls the flexor-extensor alternation of antagonistic muscle contraction (Al-Mosawie et al., 2007; Lundfald et al., 2007; Zhang et al., 2014).

The V2c are ipsilateral INs. Their differentiation depends on Sox1, a transcription factor belonging to the Group B of the Sox gene family, but also on Gata3 and Foxn4. Studies in loss of function and lineage tracing experiments revealed that this population is closely related to the V2b INs. In the *Sox1*<sup>GFP/GFP</sup> mutant mice, the number of V2c is reduced and the remaining ones are labeled by Gata3, indicating that Sox1 is essential for the production of the V2c INs. In addition, in embryos in which *eGFP* is driven by the *Gata3* promoter at e12.5, the Sox1+ V2c INs also express *eGFP*. Finally, the V2c INs are completely lost in the absence of *Foxn4* (Panayi et al., 2010). Their role in the locomotor control has not yet been elucidated.

The V2d INs are ipsilateral excitatory projecting neurons expressing *Shox2*. Although the V2d INs constitute a small population, they contribute significantly to the locomotor rhythm generation. The V2d INs are segregated in 2 subgroups, a lateral premotor population and a medial non premotor population. Premotor V2d INs directly connect to flexor MNs. The non-premotor V2d INs connect either to ipsilateral or to contralateral INs in the ventral region of the spinal cord. *Shox2* is also expressed by a V2a subpopulation that stabilize the locomotor rhythm (Figure 11) (Dougherty et al., 2013).



**Figure 11: Overview of the role of Chx10+ V2a, Chx10+ Shox2+ V2a and Shox2+ V2d INs during the locomotor control (Dougherty et al., 2013).**  
CINs = contralateral interneurons.

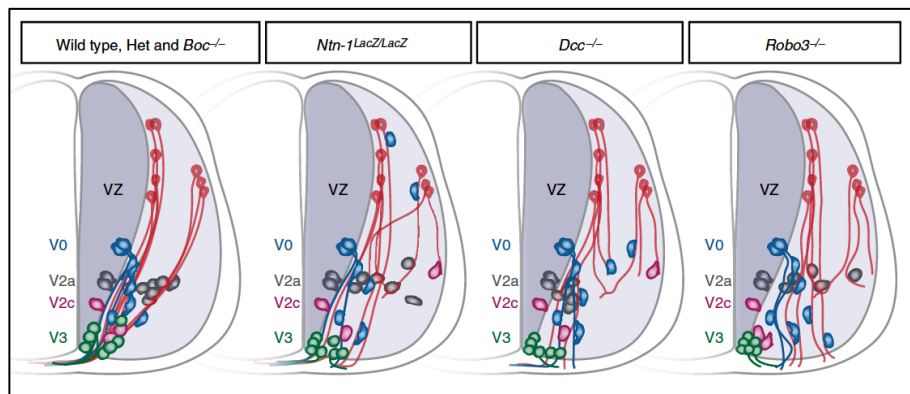
### 1.3. Diversification and migration of spinal IN populations

Although each IN population further subdivides into several subpopulations that contribute to modulate the locomotor coordination, there are more than 100 spinal IN populations at the adult stage in the vertebrates (Goulding, 2009; Grillner and Jessell, 2009). These populations seem to be derived from the 4 embryonic cardinal populations of INs described above (Arber, 2012; Lupo et al., 2006). The diversification of the cardinal IN populations is, hence, much more extensive than currently know. Recently, it has been demonstrated that the V1

INs can be further subdivided into 50 subpopulations characterized by distinct combinatorial expression of transcription factors and position in the developing spinal cord. Most of these transcription factors also subdivide the V2a in multiple subpopulations (Bikoff et al., 2016). However, the molecular and cellular mechanisms leading to the extensive diversification of ventral IN populations has not been elucidated.

In addition to their diversification, each ventral population migrates in the ventral region of the spinal cord to a very precise position in order to adequately integrate the neuronal circuitries. In the cortex, 2 different mechanisms of migration have been characterized, namely: radial migration and tangential migration. The excitatory cortical INs migrate from the ventricular zone to the cortical plate in an inside-out manner by migrating radially to the pial surface along the radial glial progenitors. This migratory mode is mostly regulated by the Reelin signaling. In contrast, the inhibitory cortical INs arise from the ganglionic eminences and migrate first tangentially to exit the ventricular zone and reach the cortical plate, then migrate radially to properly integrate the cortex. The dorsal migration of these INs are guided by attractive and repellant cues, such as Semaphorin 3A (Sema3A), Sema3F, Neuregulin-1 (NRG1), chemokine Cxcl12 and its receptor Cxcr4, Hepatocyte growth factor, brain derived neurotrophic factor (BDNF), neurotrophin 4, glial-derived neurotrophic factor (GDNF) (Huang, 2009). In the spinal cord, Reelin signaling is required for the proper position of the PGC and LMC neurons at thoracic and limb levels, respectively (Palmesino et al., 2010; Phelps et al., 2002; Yip et al., 2000). Cadherins also play an important role for sorting neurons in motor pools. Cadherin 7, a type II cadherin, and N-Cadherin, a type I cadherin, are required for proper segregation of LMCm and LMCI neurons (Chen, 2018). In addition, Reelin signaling also regulate the proper layering of the lamina I in the dorsal horn of the spinal cord (Villeda et al., 2006). However,

ventral IN migration does not seem to be regulated by Reelin signaling, and the molecular and cellular mechanisms involved in the control of ventral IN migration have been yet poorly investigated. Nevertheless, Laumonnerie et al., 2015 uncovered that the commissural axons serve as scaffold to delineate the distribution of some ventral IN populations. Indeed, the V0<sub>VCG</sub>, V2a, V2c and V3 populations are mislocated in the absence of commissural axons (Figure 12). The mislocation of V2a and V2c INs is the consequence a non-cell autonomous regulation, as these populations do not express specific marker of commissural axons (*Dcc*, *Netrin* or *Robo3*). Indeed, both the physical position of commissural axons and guidance cues produced by commissural neurons seem to influence the settling position of non-commissural IN populations (Laumonnerie et al., 2015).



**Figure 12: Schematic representation of the ventral IN distribution in different models of commissural axon disruption (Laumonnerie et al., 2015).** In Wild type embryos, the commissural axons, organized in medial and lateral fascicles, serve as scaffold for proper distribution of ventral IN population in the spinal parenchyma. In *Boc* mutant and heterozygous (Het) embryos with very mild commissural axonal defects, the ventral IN population distribution is not affected. In contrast, in mutant embryos displaying severe commissural axonal disruptions, namely *Netrin-1* (*Ntn-1*), *Dcc* and *Robo3* mutants, the ventral IN populations are mislocated in the spinal parenchyma.

More importantly, it has been demonstrated that the distribution of the ventral IN populations plays an important role on the one hand in microcircuit organization and on the other hand on their function within the CPG. Indeed,

Satb2 regulates the localization of Satb2<sup>+</sup> dorsal sensory inhibitory INs on the medio-lateral axis into the spinal cord, which is required for a proper integration in motor circuits (Hilde et al., 2016). The V1 subpopulations are regionalized along the antero-posterior axis of the spinal cord under the control of Hox genes. Indeed, Sweeney et al., 2018 have shown that *Hoxc9* and *Hoxc6* are expressed in thoracic and lumbar V1 subpopulations, respectively. Similar to MNs, the thoracic V1 subpopulations acquire the limb V1 subpopulation transcriptional identity in *Hoxc9* mutant mice, demonstrating that Hox genes mediate the regionalization of the V1 subpopulations along the antero-posterior axis. The differential distribution of each V1 subpopulation along the dorso-ventral axis of the spinal cord determines the nature of motor or sensory inputs they receive. This regionalization suggests that the V1 subpopulations in the brachial region will be involved in locomotor regulation distinct from those located at thoracic levels (Bikoff et al., 2016; Sweeney et al., 2018). Similarly, the type I and II V2a are distributed in counter-gradients along the antero-posterior axis of the spinal cord and drive unique patterns of motor activity at brachial and lumbar levels. The type I V2a INs, enriched in the lumbar region, contact the local MNs. In contrast, type II V2a INs, more numerous in the cervical spinal cord, display local and supraspinal bifurcating connections. In addition, these V2a subtypes are differentially distributed along the medio-lateral axis of the spinal cord (Figure 10) (Hayashi et al., 2018). Finally, dorsal and ventral V3 INs are functionally distinct depending on their position within the spinal parenchyma: although both dorsal and ventral V3 are recruited during running, only ventral V3 are recruited during swimming (Borowska et al., 2015; Borowska et al., 2013). Thus, the precise localization of neuronal populations in the spinal parenchyma is critical for the formation of neuronal circuits and for locomotor control.

During development, the ventral IN populations diversify and settle in very defined positions on both the dorso-ventral and the antero-posterior axis of the spinal cord. As mentioned above, few studies highlighted that the settling position of interneuron populations is critical for their input and output connectivity. This suggests that they integrate very specific local CPGs along the antero-posterior axis of the spinal cord in order to ensure locomotor control flexibility. However, the underlying molecular and cellular mechanisms involved in the regulation of ventral IN diversification and migration remain until now elusive.

### 1.4. The Onecut transcription factors

Recent studies showed that the Onecut (OC) transcription factors are expressed in the developing central nervous system and regulate different aspects of the development of specific neuronal populations, such as the production, maintenance, diversification or migration. In mammals, the OC family is composed of 3 members: Hepatocyte nuclear factor 6 (HNF-6 also called OC-1), OC-2 and OC-3 (Jacquemin et al., 1999; Lemaigre et al., 1996; Samadani and Costa, 1996; Vanhorenbeeck et al., 2002). Other species like *C. elegans* (Lemaigre et al., 1996), ascidian (Sasakura and Makabe, 2001), zebrafish (Hong et al., 2002; Matthews et al., 2008), *Xenopus* (Haworth and Latinkic, 2009), *drosophila* (Nguyen et al., 2000), sea urchin (Otim et al., 2004; Poustka et al., 2004) possess one or several homolog genes coding for OC factors.

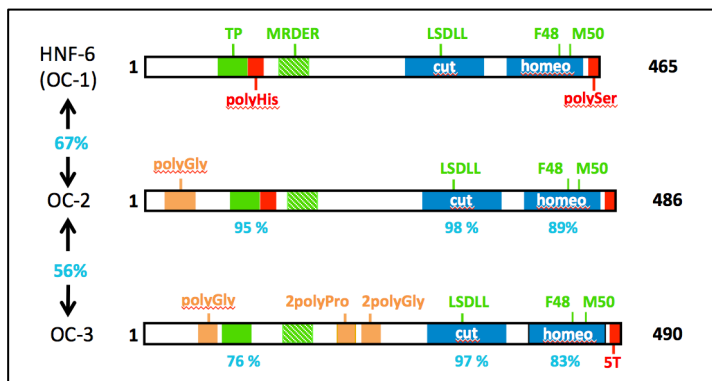
HNF-6 has been discovered during a study of the *6-phosphofructo-2-kinase* (*pfk2*) regulation in rat liver, in which they showed that HNF-6 stimulates *pfk2* expression (Lemaigre et al., 1996). *Oc2* and *Oc3* have been discovered by bioinformatic analyses based on their sequence homology with *Hnf6*. *Oc2* has been found from a fetal human retina cDNA library (Jacquemin et al., 1999), while

*Oc3* has been found by screening the human genome data banks (Vanhorenbeeck et al., 2002).

#### **1.4.1. Onecut factor structure**

The sequence of the OC transcription factors comprises several regions highly conserved between the 3 OC proteins. Indeed, the OC factors are characterized by a bipartite DNA-binding domain: a cut domain and a divergent homeodomain separated by a short flexible linker of 27 amino acids. The 3D structure of the unique cut domain is organized in 5  $\alpha$ -helices (helices 1-3 and 5-6) and a small  $3_{10}$  helix (helix 4), which is defined by 3 residues per turn and 10 atoms in the rings forming by the hydrogen bounds. A  $3_{10}$  helix is characterized by the presence of a hydrogen bound between the amino terminal of a residue and the carboxy terminal of the first of 3 residues, the repetition of these bounds defines the  $3_{10}$  helix. The third helix, called the « recognition helix », binds to the target DNA. This domain also contains a LXXLL motif in the recognition helix capable to interact with co-factors. The homeodomain is organized in 3  $\alpha$ -helices (helices 7-9) and binds to the DNA with the helix 9, the recognition helix (Iyaguchi et al., 2007). The homeodomain differs from other homeodomains by the presence in the recognition helix of a phenylalanine and a methionine at positions 48 and 50 respectively, called the F48M50 dyad, which is never seen in classical homeodomains. Other highly conserved regions compose the sequence of the OC factors. A sequence rich in serine, threonine and proline of 24 amino acids, the TP box, is found in HNF-6 and OC-2 protein. Although present in OC-3, this box does not contain serine residues. A polyhistidine tract upstream of the cut domain, and a serine-rich carboxy-terminal region of 19 amino acids are also present in HNF-6 and OC-2. These tracts are not present in OC-3, however OC-3 contains 5

threonine residues in the carboxy-terminal, 2 polyproline and 3 polyglycine tracts. Finally, OC-2 also contains a polyglycine tract upstream of the TP box, as OC-3 (Figure 13) (Jacquemin et al., 1999; Vanhorenbeeck et al., 2002).



**Figure 13: Schematic representation of OC factor conserved sequences.**

In green are represented the residues or the regions that stimulate the transcriptional activity of OC factors, in red those that inhibit it, finally in orange those for which the activity has not been

investigated. The percentage of identity between the conserved regions (compared to OC-1) and between the 3 OC proteins is also annotated.

HNF-6 exists as 2 isoforms, HNF-6 $\alpha$  and HNF-6 $\beta$ , generated by alternative splicing. HNF-6 $\beta$  differs from HNF-6 $\alpha$  only by the presence of an additional sequence of 26 amino acids in the linker between the cut domain and the homeodomain. These 2 isoforms recognize the same target genes, however, their affinity and their DNA-binding kinetic differ according to the target genes. *Hnf6 $\alpha$*  seems to constitute the predominant isoform (Lannoy et al., 1998).

#### 1.4.2. Transcriptional activity

The OC transcription factors have been characterized as transcriptional activators. The cut domain and the homeodomain constitute their DNA-binding regions. Studies have tested by EMSA (Electrophoretic mobility shift assay) the ability of deletion mutants of Hnf6 to bind to 2 different target genes, namely: Hepatocyte nuclear factor-3 $\beta$  (*Hnf3 $\beta$* ) and Transthyretin (*ttr*). It has been



demonstrated that HNF-6 binds to the DNA according 2 distinct mechanisms. Indeed, only the cut domain is necessary for binding to *Hnf3 $\beta$*  gene, while both DNA-binding domain are required for binding to *ttr* gene. Thus, the cut domain is essential for the DNA binding of OC proteins. In contrast, the homeodomain is unable to bind alone to the DNA. However, it increases the DNA-binding specificity and selectivity of the OC factors to their target genes when its binding is not required (Lannoy et al., 1998). Other target genes were identified based on the consensus sequence 5' (A/T/G)-(A/T)-(A/G)-T-C-(A/C)-A-T-N-(A/T/G) 3' derived from the characterization of the *Hnf3 $\beta$*  and the *ttr* regulatory sequences. Among 6 target sequences, 5 of them contain the TCAAT core conserved in the majority of the recognition sequence of OC factors. The affinity of OC factors for these targets was tested by EMSA (Table 1) (Jacquemin et al., 2001; Lannoy et al., 1998; Samadani and Costa, 1996; Vanhorenbeeck et al., 2002).

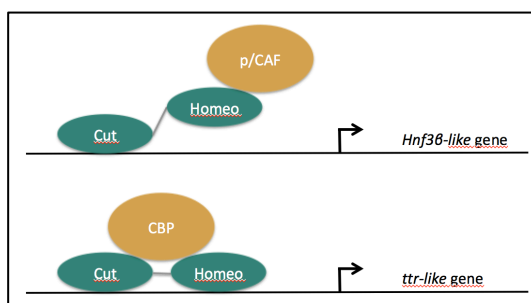
| Target genes | Recognition sequences              | HNF-6 | OC-2 | OC-3 |
|--------------|------------------------------------|-------|------|------|
| Hnf3 $\beta$ | GAAAAAA <b>AAATCAATAT</b> CGGGCCT  | +++   | ++   | +++  |
| TTR          | GTCTGCT <b>AAGTCAATAAT</b> CAGAAAT | +++   | +    | +    |
| Hnf4         | AGGATAG <b>AAGTCAATGAT</b> CTGGGA  | +     | -    | ++   |
| Glucokinase  | GGAAAG <b>TGATCAATCG</b> TGTCAAG   | +++   | ++   | +    |
| Mitf         | AAAAATA <b>AAATCAACAT</b> TTAA     | +++   | ++   | +    |
| Pdx1         | AGGCATG <b>TAATCAATAA</b> ATAACAT  | ++    | ++   | +    |
| Mutated Pdx1 | AGGCATG <b>TAACAAATAA</b> ATAACAT  | -     | -    | -    |

**Table 1: OC factor recognition sequences on different target genes (adapted from (Vanhorenbeeck et al., 2002)).** The recognition sequences on 6 target genes (*Hnf3 $\beta$* , *TTR*, *Hnf4*, *Glucokinase*, *Mitf*, *Pdx1* and mutated *Pdx1*) are represented in bold in the second column. In the third column, the + and – represent the affinity of each OC factor for each recognition sequence.

The transcriptional role of some conserved regions of the OC proteins have been characterized by directed mutagenesis. The transcriptional activity of OC

factors was assessed by co-transfections of expression vector for *Hnf6* mutants (point mutations) and reporter constructs containing a luciferase reporter gene under the control of regulatory regions bound by HNF-6. It has been shown that the TP box, the F48M50 dyad and the LXXLL motif stimulate the transcriptional activity of OC factors. In contrast, the polyhistidine and the polyserine tracts inhibit this activity (Lannoy et al., 2000).

Furthermore, cofactors able to interact with OC proteins have been identified. Indeed, when both cut domain and homeodomain are required for binding to the DNA, the coactivator CREB-binding protein (CBP) is recruited and interacts with the LXXLL motif of the cut domain and the F48M50 dyad of the homeodomain to stimulate the transcriptional activity of OC factors. In contrast, when only the cut domain is required for DNA-binding, the coactivator p300/CBP-associated factor (p/CAF) interacts with the OC factors in an LXXLL-independent manner (Figure 14) (Lannoy et al., 2000). CBP and p300/CAF are histone acetyltransferase proteins that stimulate gene transcription by acetylating lysine residues on histones. Histone acetylation causes their partial dissociation from the DNA, making DNA regulatory sequences more accessible for transcription factors. CBP acetylates also HNF-6 to increase its transcriptional activity (Rausa et al., 2004; Tanaka et al., 2000).



**Figure 14: Control of *Hnf3β-like* and *ttr-like* expression according to 2 distinct mechanisms.**

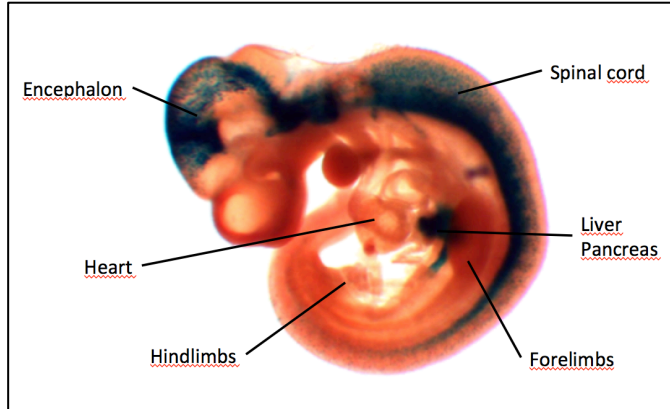
For the stimulation of *Hnf3β-like* gene expression, only the cut domain is necessary for binding to the DNA, while the homeodomain interact with the p/CAF cofactor. For the stimulation of *ttr-like* gene expression both domains are required for binding to the *ttr* gene and for interacting with the CBP cofactor.

OC factors bind the DNA as a monomer. When the cut domain and the homeodomain are required for DNA-binding, both domains wrap and bind the target DNA by inserting their recognition helices into the major groove of the DNA. Instead, only the recognition helix of the cut domain contacts the major groove of the DNA when the homeodomain is not required for binding (Iyaguchi et al., 2007; Lannoy et al., 1998).

### 1.4.3. Pattern of expression and roles of OC factors

The OC factors display a partially overlapping expression pattern characterized by a spatial HNF-6>OC-2>OC-3 hierarchy. During the development, they are dynamically expressed over time and diminished in adulthood. The OC factors are not expressed in entire cell populations but in subsets of cells. They are either transiently expressed, or continuously, without any apparent correlation between the expressing populations. The overlapping pattern of expression and the high sequence homology between the 3 OC proteins suggest that they could exert similar and even redundant functions during the development. Indeed, the simple mutant mice for each OC factor exhibit a milder phenotype than the double mutants for OC (*Hnf6*<sup>-/-</sup>; *Oc2*<sup>-/-</sup>, *Hnf6*<sup>-/-</sup>; *Oc3*<sup>-/-</sup>, *Oc2*<sup>-/-</sup>; *Oc3*<sup>-/-</sup>), which die at birth. However, among the OC mutants, the *Hnf6* mutant mice is more affected than *Oc2* mutants that is in turn more affected than *Oc3* mutants, which appear normal.

The OC factors are expressed in the endoderm, in endoderm derivatives and in the nervous system (Figure 15).



**Figure 15: Expression pattern of OC-2 in a mouse embryo.**

Labeling of the  $\beta$ -galactosidase activity on an  $Oc2^{lacZ/+}$  mouse embryo (picture: Jacquemin P.). This labeling recapitulates the expression pattern of 3 OC factors.

In the liver and the pancreas, OC are present in progenitors and in differentiated cells and play critical roles during their development or specification by controlling the expression of genes, such as: *Pdx1*, *Ngn3*, *Hnf3 $\beta$* , *PFK2*, *PEPCK*, *glucokinase*, *Hnf1 $\beta$* , or by controlling the TGF $\beta$ /activin signaling (Clotman et al., 2005; Clotman et al., 2002; Gannon et al., 2000; Jacquemin et al., 2000; Jacquemin et al., 1999; Jacquemin et al., 2003a; Landry et al., 1997; Margagliotti et al., 2007; Pierreux et al., 2006; Pierreux et al., 2004; Vanhorenbeeck et al., 2002; Vanhorenbeeck et al., 2007; Zhang et al., 2009). In the liver, HNF-6 and OC-2 control the hepatoblast migration. The liver is initially formed by a hepatic bud derived from the ventral foregut endoderm. The hepatoblasts then proliferate and migrate towards the septum transversum mesenchyme where they differentiate into hepatocytes or biliary cells. Margagliotti et al., 2007 have shown that HNF-6 and OC-2, expressed at the onset of the liver development, are not required for hepatoblast proliferation but control redundantly their migration towards the mesenchyme by stimulating the degradation of the basal lamina surrounding the liver bud. The characterization of the *Hnf6 $^{-/-}$* ; *Oc2 $^{-/-}$*  double mutant phenotype highlighted that the OC factors control the expression of *E-cadherin*, *Thrombospondin* and *Osteopontin* involved in the

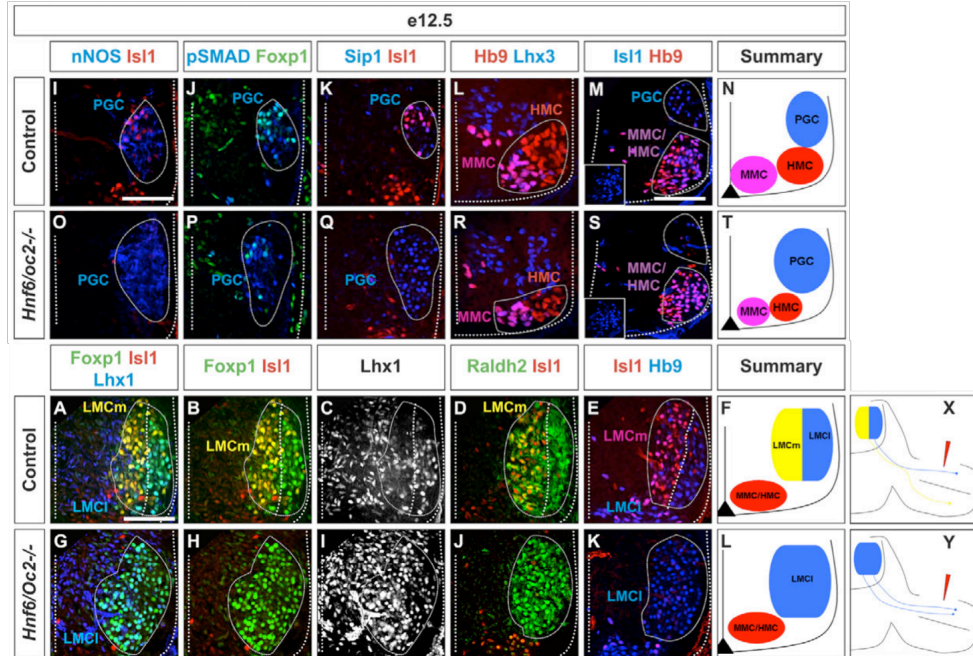
control of hepatoblast adhesion and migration (Margagliotti et al., 2007). Oc2 and Oc3 are also expressed in the stomach and the intestine (Jacquemin et al., 1999; Maier et al., 2006; Vanhorenbeeck et al., 2002). Finally, Oc2 is also present in the melanocytes (Jacquemin et al., 2001; Jacquemin et al., 1999). The expression of *Hnf6* and *Oc2* is regulated by some micro-RNA (miR). In the liver and the pancreas, miR-495 and miR-218 repress *Hnf6* and *Oc2* expression, respectively (Simion et al., 2010). In liver, HNF-6 and miR-122 mutually stimulate their expression in a positive feedback loop (Laudadio et al., 2012).

In the CNS, the OC factors are expressed by differentiating neuronal populations. Their role during the differentiation and migration of hepatic and pancreatic cells led to investigate their role during the CNS development. Several recent studies demonstrated that OC factors are also key determinants during these processes in specific neuronal populations.

OC factors control the production of a specific part of meso-diencephalic dopaminergic (mdDA) neurons, in which they are expressed at early stages of their development. The mdDA neurons are critical for essential brain function, namely associative motor learning, emotion and reinforcement, as well as motor control (Chakrabarty et al., 2012). OC factors are also required for the production of the rhombencephalic part of the mesencephalic trigeminal nucleus (MTN), in which they are continuously expressed during their development. The MTN is a sensory nucleus in the encephalon implicated in the integration of the reflex control of oral and facial movements. In the absence of OC factors, the MTN is completely lost in the hindbrain, while the mesencephalic part of the MTN is preserved (Espana and Clotman, 2012a). Finally, OC factors are required for the production of horizontal cells in the retina. Different cell types arise from the

retinal precursor cells, including horizontal cells. During the development, *Hnf6* and *Oc2* are expressed in both retinal precursor cells and horizontal cells. The absence of OC factors results in a complete loss of horizontal cells (Sapkota et al., 2014; Wu et al., 2012). Taken together, this data showed that the OC factors are necessary for the production of 3 specific neuronal populations in the encephalon.

In the spinal cord, studies led by Roy et al., 2012 have demonstrated that OC factors are required for proper diversification of MNs, in which they are differentially and dynamically expressed in different subsets during their development (Francius and Clotman, 2010). Indeed, the OC proteins directly control the *Is/1* expression by binding to their recognition site within the CREST2 enhancer, part of the *Is/1* regulatory sequences (Kim et al., 2015; Uemura et al., 2005). Hence, they control at thoracic level of the spinal cord, the differentiation ratio between the somatic and visceral MNs. In the absence of OC factors, the visceral MNs are produced in excess at the expense of somatic MNs (Figure 16). At limb levels, the OC factors control the LMCm/LMCI ratio. Indeed, although the total number of the LMC neurons is not affected in the absence of OC factors, the LMCm neurons are depleted and converted into LMCI neurons. This identity switch is accompanied by a redirection of the corresponding axons towards the dorsal mesenchyme of the limbs (Figure 16). Indeed, the loss of *Is/1* expression results in a loss of *Ephb1* expression that is essential to guide the LMCm axons towards the ventral limb mesenchyme (Roy et al., 2012).

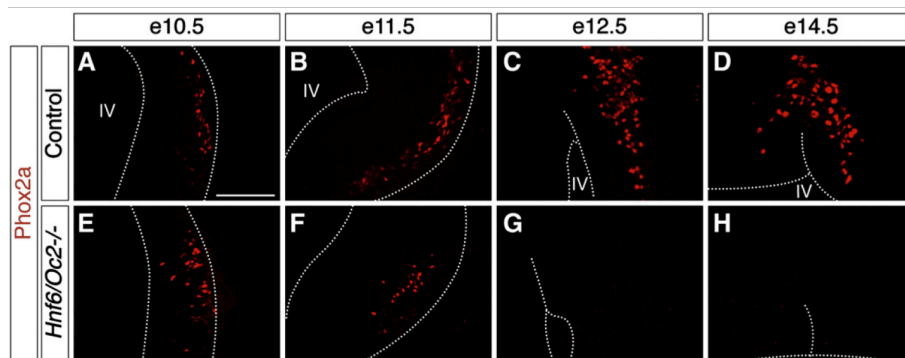


**Figure 16: The OC factors control the diversification of MNs (figure from Roy et al., 2012).** (I-T) At thoracic level, OC factors control the differentiation balance between the somatic (HMC and MMC) and visceral (PGC) MNs. In the absence of OC factors, the visceral MNs are produced in excess at the expense of somatic MNs. (A-L) At limb levels, OC factors promote the diversification of the LMCm neurons and control the LMCm/LMCI ratio. In the absence of OC factors, the LMCm neurons are depleted and converted into LMCI neurons, while the total number of the LMC neurons is not affected. (X-Y) This molecular identity switch is accompanied by a redirection of the LMC axons towards the dorsal mesenchyme of the limbs.

Besides MNs, the OC factors are also differentially and dynamically expressed in postmitotic dorsal INs. They are expressed in subsets of each dorsal population, except the dl1 INs. During their development, the OC factors regulate the diversification of the dl5 subpopulation containing *Phox2a*. In OC-deficient mutant mouse embryos, the number of *Phox2a*+ dl5 INs significantly decrease at thoracic level of the spinal cord (Kabayiza et al., 2017).

The OC factors also appears to be necessary not for the production, but for the maintenance of neuronal populations. In the encephalon, OC factors control the proper migration and the maintenance of the noradrenergic phenotype of the

Locus Coeruleus (LC), or A6 nucleus, although being only transiently expressed at the onset of its development. Located in the dorsal part of the pons in close vicinity of the MTN, the LC is the main noradrenergic nucleus of the CNS and is involved in the regulation of sleep-awakeness cycle, arousal, attention, anxiety and adaptative behaviors. In the absence of OC proteins, the LC neurons are produced in normal numbers, however this nucleus is not maintained later in the development (Figure 17). The OC factors being only transiently expressed in these neurons, this suggests that OC regulate in a non-cell autonomous manner the maintenance of the LC during the development, possibly via the rhombencephalic portion of the MTN, which is located in close vicinity of the LC in control individuals and is absent in *Oc* mutants (Espana and Clotman, 2012a).

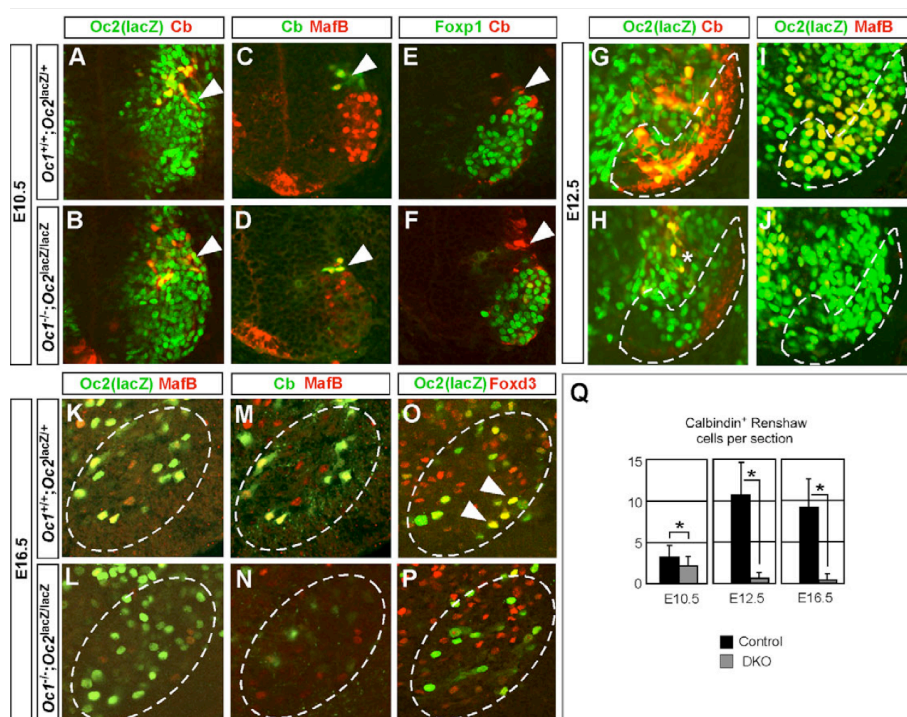


**Figure 17: The OC factors control the maintenance of the noradrenergic phenotype of the Locus Coeruleus (figure from Espana and Clotman, 2012a).** (A-D) The LC neurons, characterized by *Phox2a* expression, migrate radially to the pial surface after their production. Then, they migrate ventrally and caudally to form a compact population adjacent to the fourth ventricle (IV). (E-H) In the absence of OC factors, although well produced, the LC neurons are mislocated to progressively lose their molecular identity over time.

In addition, in the spinal cord, the OC proteins are necessary for maintaining the differentiation program of RCs, in which they are continuously present during development. Indeed, the development of RCs, characterized by successive expression of *MafB* and *Calbindin*, is well initiated in the *Hnf1b*<sup>-/-</sup>;*Oc2*<sup>-/-</sup>



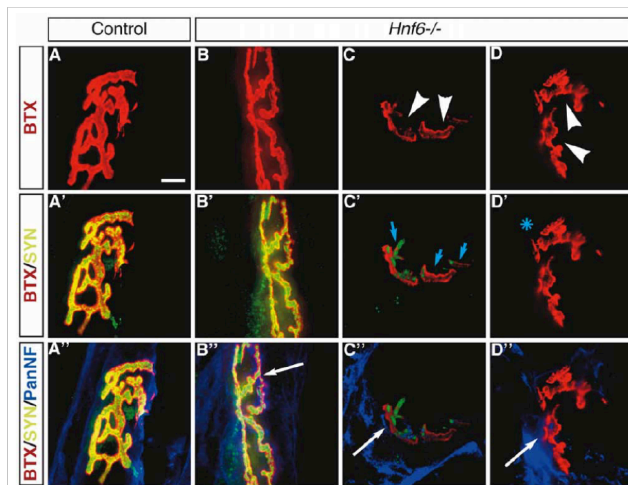
compound mutant mouse embryos but the RCs are rapidly depleted over time. This depletion is accompanied by the lack of RC axons normally present in the VLF (Figure 18) (Stam et al., 2012). Taken together, these studies show that the OC factors control the maintenance of these neuronal populations in 2 distinct ways: cell-autonomous or non-cell autonomous manner.



**Figure 18: The OC factors maintain the differentiation program of Renshaw cells (figure from Stam et al., 2012).** (A-F, Q) At e10.5, the RC differentiation program, comprising Calbindin (Cb), MafB and OC2, is well initiated in the absence of OC factors, although a small reduction of the cell number is observed. (G-I, Q) At e12.5, the RC are almost completely lost with some ectopic cells within the MN area in the *Hnf6*<sup>-/-</sup>; *Oc2*<sup>-/-</sup> compound mutant embryos. The depletion is accompanied by the lack of the RC axons in the VLF. (K-Q) At e16.5, the loss of RCs is confirmed in OC mutant embryos.

The OC factors also control connectivity of some neuronal populations. Indeed, OC-2, regulated by BMP4 signaling, controls the central projection of the trigeminal ganglion neurons towards the brainstem. These central projections are necessary for transmitting the spatial information of the face into spatial maps

within the brain (Hodge et al., 2007). Similarly, HNF-6 controls the formation of the neuromuscular junctions (NMJ). The NMJ are specialized synapses whereby MNs of the spinal cord control the contraction of skeletal muscles. Audouard et al., 2012 have showed that the *Hnf6* mutant mice exhibit hindlimb locomotor defects that result from muscle weakness and wrong position of the hindlimbs. It appeared that HNF-6 regulates the formation and the maturation of the NMJs by controlling the expression of *Agrin*, *Neuregulin-2* and *TβRII* in MNs, all involved in NMJ formation. In addition, HNF-6 regulates the distribution of synaptophysin, the MN presynaptic vesicular marker (Figure 19). Hence, HNF-6 regulates a genetic program involved in NMJ formation (Audouard et al., 2012). Taken together, these studies demonstrate that the OC factors are required for proper neuronal connectivity of specific neuronal populations.

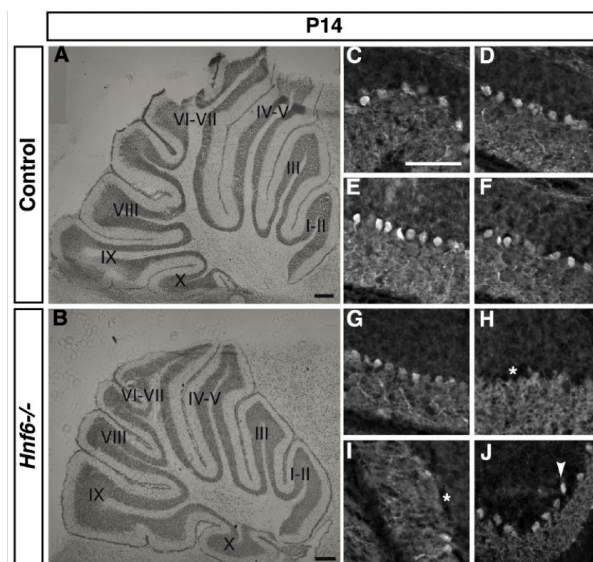


**Figure 19: HNF-6 controls the formation and the maturation of neuromuscular junctions (figure from Audouard et al., 2012).** (A-A'') In control embryos, NMJ display a pretzel-like shape. They are characterized by the superposition of  $\alpha$ -Bungarotoxin (BTX), a marker of the motor endplate, synaptophysin (SYN) and neurofilament (PanNF), markers of the MN terminals and axons, respectively. (B-D'') In the absence of OC factors, the majority of the NMJs fails to organize in a pretzel-like shape. In addition, synaptophysin is

either fragmented and not superposed to BTX, or absent. However, the NMJs are well innervated, as evidenced by the neurofilament labeling.

Finally, as it is the case for the hepatoblasts in the developing liver (Margagliotti et al., 2007), OC factors control the distribution and the migration of several neuronal populations. In the cerebellum, *Hnf6* is transiently expressed in the Purkinje cells after birth. These cells contribute to the cerebellum function,

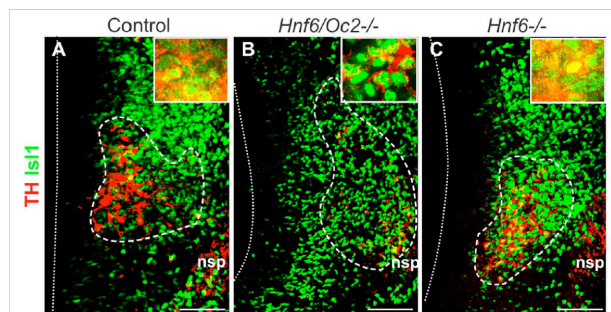
namely the fine control of a balanced and coordinated locomotion. HNF-6 is required for proper reorganization of Purkinje cells from a multilayered organization into a mature monolayer. The absence of *Hnf6* results in abnormal reorganization with superpositions or absence of Purkinje cells at the interface between the molecular and the granular layer (Figure 20). This phenotype is likely responsible for the balance and coordination defects observed in the *Hnf6* mutant mice (Audouard et al., 2013).



**Figure 20: The OC factors are required for the proper organization of Purkinje cells into a monolayer (figure from Audouard et al., 2013).** (A, C-F) In control mice, the Purkinje cells are organized in a regular and aligned monolayer. (B) In the absence of OC factors, the cerebellum is smaller than in control mice. (G-J) The Purkinje cells are properly organized in monolayer at some locations, however, they are either superposed, not aligned, or absent at some other locations.

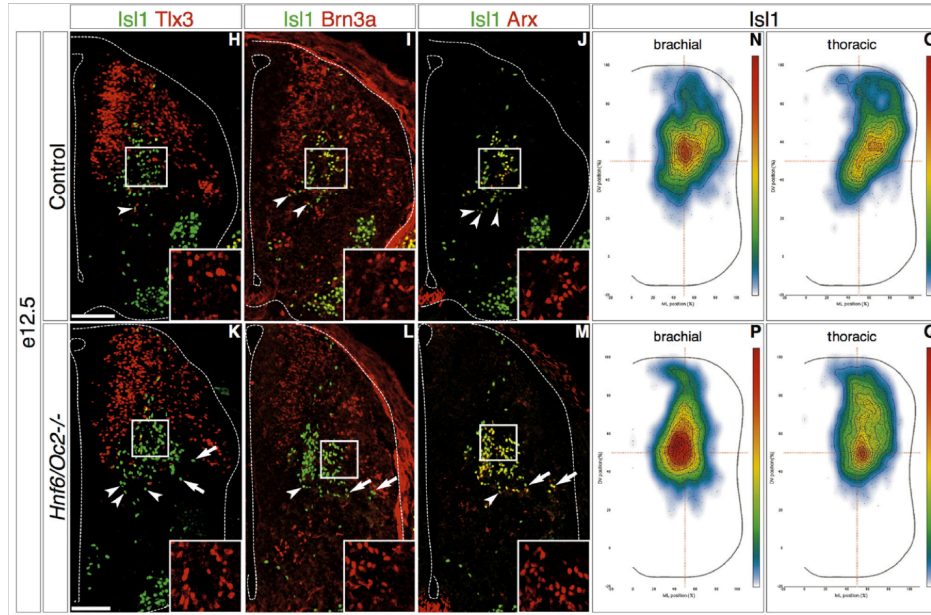
The OC factors control migration of the A13 neurons during the second phase of their development. Located in the ventral thalamus, the A13 nucleus is the largest diencephalic dopaminergic population. These neurons exert an autonomous role by integrating sensory input to autonomic, neuroendocrine and motor outputs. The development of the A13 nucleus occurs in 2 phases: first, the A13 precursors differentiate into dopaminergic neurons during their radial migration towards the pial surface, then the A13 neurons migrate and gather in

the vicinity of the third ventricle. The OC factors are dynamically and differentially expressed during the development of the A13 nucleus. Studies in the *Hnf6*<sup>-/-</sup>;*Oc2*<sup>-/-</sup> double mutant showed that A13 neurons are more scattered in the absence of OC factors and that they are located farther from the third ventricle. This suggest that OC factors control the A13 neuron adherence to keep them organized as a nucleus (Figure 21) (Espana and Clotman, 2012b).



**Figure 21: The OC factors control the distribution of A13 neurons (figure from Espana and Clotman, 2012b).** In control (A) and *Hnf6*<sup>-/-</sup> (C) embryos, the A13 neurons, characterized by the expression of *TH* and *Isl1*, form a compact nucleus in the vicinity of the third ventricle (left to the dotted lines). (B) In the *Hnf6*<sup>-/-</sup>;*Oc2*<sup>-/-</sup> double mutant embryos, the A13 neurons are more scattered and fail to reach the vicinity of the third ventricle.

In the spinal cord, Kabayiza et al., 2017 have shown that the OC factors control the distribution of the dI3 and dI5 INs. Indeed, in the absence of OC factors, these populations are produced in comparable number of cells to the control embryos, however, their distribution is altered within the spinal parenchyma (Figure 22) (Kabayiza et al., 2017).



**Figure 22: The OC factors control the distribution of dI3 (figure from Kabayiza et al., 2017).** (H-J, N-O) In control embryos at e12.5, the dI3 INs migrate in dorso-lateral to ventro-medial direction with the density peak in the center of the population. (K-M, P-Q) In the absence of OC factors, the dI3 INs migrate in a dorso-medial to ventro-medial direction with the density peak moved close to the migration front of the population.

Hence, all these studies demonstrated that the OC factors are key determinants during the development of multiple neuronal populations (table 2), as they do during the development of the liver and the pancreas. By controlling the production, diversification, maintenance, connectivity and settling position of specific neuronal populations, the OC factors likely contribute to their proper integration in neuronal circuits, essential to their proper functioning. Although their mechanisms of action are poorly understood, they seem different from those highlighted in the developing liver and pancreas. Indeed, some OC targets, namely *Neurogenin-3*, *Pdx1*, *Hnf1 $\beta$* , *Ptf1a* and *Hnf-4*, which are also present in the CNS, do not seem to be downstream of OC factors during neural development. Furthermore, the regulation of OC factors has been poorly investigated in the CNS. Nevertheless, studies in chicken embryos have demonstrated that miR-9,

strongly expressed in the LMCI, represses *Hnf6* expression in this motor column resulting in a weak *Hnf6* expression, while it is strongly expressed in LMCm neurons where miR-9 is not present (Luxenhofer et al., 2014; Radhakrishnan and Alwin Prem Anand, 2016).

| Function              | OC    | Neuronal population | Target                         | Mechanism                  | Reference                   |
|-----------------------|-------|---------------------|--------------------------------|----------------------------|-----------------------------|
| Production            | All   | mdDA neurons        | ?                              | ?                          | (Chakrabarty et al., 2012)  |
|                       | All   | MTN                 | ?                              | ?                          | (Espana and Clotman, 2012a) |
| Diversification       | All   | MNs                 | Isl1                           | Direct binding             | (Roy et al., 2012)          |
|                       | All   | Phox2a+ dI5         | ?                              | ?                          | (Kabayiza et al., 2017)     |
| Maintenance           | All   | RC                  | ?                              | ?                          | (Stam et al., 2012)         |
|                       | All   | LC neurons          | ?                              | Non-cell autonomous?       | (Espana and Clotman, 2012a) |
| Neuronal connectivity | OC-2  | TG neurons          | ?                              | ?                          | (Hodge et al., 2007)        |
|                       | HNF-6 | NMJ                 | Agrin, TGFbRII<br>Neuregulin-2 | Direct, indirect binding ? | (Audouard et al., 2012)     |
| Migration             | HNF-6 | Purkinje cells      | ?                              | ?                          | (Audouard et al., 2013)     |
|                       | All   | A13 neurons         | ?                              | ?                          | (Chakrabarty et al., 2012)  |
|                       | All   | dI3, Pox2a+ dI5     | ?                              | ?                          | (Kabayiza et al., 2017)     |

Table 2: Overview of the different roles of OC factors during the central nervous system development.

The OC factors being also expressed in subsets of each ventral IN population, suggest that they might contribute to diversification and/or migration of these neurons in order to ensure their proper integration in CPG in the ventral region of the spinal cord.



## **2. Objectives and strategies**

In the ventral spinal cord, the OC factors are expressed in subsets of each V2 population (Francius and Clotman, 2010). This suggests that OC could contribute to the control of the V2 population diversification and/or migration as in MNs and in dorsal IN populations.

The 2 main questions of my thesis were: what are the OC factors roles during the V2 IN development? By which mechanism do they control the V2 IN development? Therefore, the aims were first to identify subpopulations within each ventral IN population. Second, to identify V2 subpopulations in which the OC factors are present. Third, to characterize the OC factor roles during the diversification and the migration of each population and subpopulation of V2 INs wherein OC factors are present. Fourth, to elucidate the molecular and cellular mechanisms involved in the regulation of the V2 IN diversification and migration. Finally, to characterize the roles of selected candidates downstream of OC factors during the V2 population development.

To do this, we first identified subpopulations in each ventral IN population by characterizing the expression of transcription factors previously described in the ventral region of the embryonic spinal cord in combination with the specific markers of each IN population. Based on that, we established the expression profile of OC factors in each identified subpopulation. To study the roles of OC factors in the V2 population diversification and migration, we used transgenic mice deficient for *Hnf6* and *Oc2* (Clotman et al., 2005; Jacquemin et al., 2000). In this model, *Oc3* expression is also lost in the spinal cord (Roy et al., 2012), enabling us to characterize the loss of OC factors without any compensation from *Oc3*. The *Oc* mutants allowed us to study different aspects of the V2 IN



development and therefore to establish the roles of OC factors in this population. On the one hand, to study the diversification of V2 populations into subpopulations, we compared the number of cells composing each population and subpopulation in control or *Hnf6*<sup>-/-</sup>; *Oc2*<sup>-/-</sup> mutant embryos. On the other hand, we adapted a quantitative evaluation method of MNs distribution into the spinal cord to compare the distribution of V2a and V2b populations and subpopulations in the presence or absence of OC factors (Palmesino et al., 2010). To elucidate the molecular and cellular mechanisms involved in the control of V2 differentiation and migration by the OC factors, we compared the transcriptome of control or *Oc* mutant neural tubes. Detailed analysis of microarray results allowed identifying candidate genes downstream of OC factors, including *Pou2f2* and *Nkx6.2*, which are differentially expressed in the mutants. Based on that, we studied the expression profile and the distribution of these 2 candidate genes in the ventral region of the spinal cord in control or in OC-deficient mutant embryos. Finally, we determined the possible implication of *Pou2f2* and *Nkx6.2* in these processes by using loss- or gain-of-function experiments. On the one hand, we studied the consequences of the loss of *Pou2f2* or *Nkx6.2* on V2 diversification and distribution by using *Pou2f2* or *Nkx6.2* mutant mouse embryos (Corcoran et al., 1993; Southwood et al., 2004). On the other hand, complementary to the loss-of-function experiments, we characterized the overexpression of *Pou2f2* by *in ovo* electroporation of an expression vector for *Pou2f2* in embryonic chick spinal cord. The role of *Pou2f2* after overexpression was assessed as for the mouse model.

### **3. Results**

#### **3.1. Identification of subsets of ventral interneurons and distribution along the rostrocaudal axis of the developing spinal cord**

##### **3.1.1. Foreword**

The ventral neuronal populations are organized in circuits that finely regulate different motor tasks all along the antero-posterior axis of the spinal cord. This suggests that these populations diversify and are regionally organized as it has been extensively studied for the MNs. However, although hundreds of distinct cell types have been identified in the adult spinal cord, the diversification of ventral IN populations in embryonic spinal cord has been yet poorly investigated.

In this study, we identified several subpopulations in each ventral IN population based on the expression of different transcription factors previously identified in the ventral region of the developing spinal cord. In addition, we characterized the distribution of each ventral population and subpopulation along the antero-posterior axis in order to assess whether they are regionally organized as MNs are.

We provide evidence that the ventral IN populations are regionally distributed along the antero-posterior axis of the spinal cord and that they further subdivide into several subpopulations.

This work was published in PLOS ONE the 15<sup>th</sup> of August 2013. I personally contribute to the characterization of the V0 and V3 IN diversification (Figures 24 and 28)

**Identification of multiple subsets of ventral interneurons and differential distribution along the rostrocaudal axis of the developing spinal cord**

**Short Title: Ventral interneuron distribution & subset diversity**

Cédric Francius<sup>1</sup>, Audrey Harris<sup>1</sup>, Timothy J. Hendricks<sup>2</sup>, Floor J. Stam<sup>2</sup>, Melissa Barber<sup>3</sup>, Dorota Kurek<sup>4</sup>, Frank G. Grosveld<sup>4</sup>, Alessandra Pierani<sup>3</sup>, Martyn Goulding<sup>2</sup> and Frédéric Clotman<sup>1\*</sup>

<sup>1</sup>*Université catholique de Louvain, Institute of Neuroscience, Laboratory of Neural Differentiation, 55 avenue Hippocrate (B1.5511), B-1200 Brussels, Belgium.*

<sup>2</sup>*Molecular Neurobiology Laboratory, The Salk Institute for Biological Studies, 10010 North Torrey Pines Road, La Jolla, CA 92037, USA.*

<sup>3</sup>*CNRS UMR 7592, Institut Jacques Monod, Université Paris Diderot, Sorbonne Paris Cité, 75205 Paris Cedex 13, France.*

<sup>4</sup>*Erasmus MC Stem Cell Institute, Department of Cell Biology, Erasmus Medical Center, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands.*

\* Corresponding author: frederic.clotman@uclouvain.be

**Corresponding author:** Frédéric Clotman, Laboratory of Neural Differentiation, Institute of Neuroscience, Université catholique de Louvain Avenue Hippocrate 55, box B1.55.11, B-1200, Brussels, Belgium  
Phone: +32.2.764.55.71, Fax: +32.2.764.55.72, Email: frederic.clotman@uclouvain.be

### Abstract

The spinal cord contains neuronal circuits termed Central Pattern Generators (CPGs) that coordinate rhythmic motor activities. CPG circuits consist of motor neurons and multiple interneuron cell types, many of which are derived from four distinct cardinal classes of ventral interneurons, called V0, V1, V2 and V3. While significant progress has been made on elucidating the molecular and genetic mechanisms that control ventral interneuron differentiation, little is known about their distribution along the antero-posterior axis of the spinal cord and their diversification. Here, we report that V0, V1 and V2 interneurons exhibit distinct organizational patterns at brachial, thoracic and lumbar levels of the developing spinal cord. In addition, we demonstrate that each cardinal class of ventral interneurons can be subdivided into several subsets according to the combinatorial expression of different sets of transcription factors, and that these subsets are differentially distributed along the rostrocaudal axis of the spinal cord. This comprehensive molecular profiling of ventral interneurons provides an important resource for investigating neuronal diversification in the developing spinal cord and for understanding the contribution of specific interneuron subsets on CPG circuits and motor control.

### **3.1.2. Introduction**

Over the past two decades, an outline of molecular mechanisms that generate neuronal diversity in the developing spinal cord, coupled with a greater understanding of how these neurons are assembled into functional circuits, has begun to emerge. Repetitive motor activities, including swimming or walking, are controlled by complex central pattern generator (CPG) networks (Berkowitz, 2001; Cazalets, 2000; Cazalets et al., 1995; Kjaerulff and Kiehn, 1996). Spinal CPG networks generate and coordinate the rhythmic and stereotyped patterns of motor activity independently of sensory or motor inputs (Armstrong, 1986; Grillner, 2003; Kiehn, 2006). These CPGs are broadly composed of motor neurons (MNs), the effectors of motor actions that are organized into functional motor pools, and several types of interneurons (INs) that serve to coordinate MN activity within and between CPG modules (Cazalets, 2000; Garcia-Campmany et al., 2010; Jankowska, 2001a, 2008; Jordan et al., 2008; Kiehn, 2006; Kyriakatos et al., 2011). However, the topographic distribution of these different IN types along the rostrocaudal axis of the spinal cord remains poorly characterized, and the diversification of IN subsets that constitute the CPG has been only partially studied.

Among the neuronal populations that make up the spinal CPG circuitry, MNs are the component most well characterized (for review (Dalla Torre di Sanguinetto et al., 2008)). MN diversification along the rostrocaudal axis is regulated by extrinsic signals that include retinoic acid (RA), fibroblast growth factors (FGFs), Wnt proteins and GDF11 (Liu, 2006; Liu et al., 2001; Nordstrom et al., 2006). Combined activity of Hox transcription factors shapes the spinal cord along the rostrocaudal axis into a brachial, thoracic and lumbar regions (Carpenter, 2002; Chanut, 2006; Diez del Corral and Storey, 2004; Krumlauf et al., 1993). In each of these regions, MN diversify into distinct subclasses which form several columns according to a

Hox code (Dasen et al., 2008; Dasen et al., 2003; Liu et al., 2001; Rousso et al., 2008). At brachial levels, combined activities of Hoxc6 and Hoxc8 specify MNs into lateral motor column (LMC) (Dasen and Jessell, 2009b; Dasen et al., 2003; Dasen et al., 2005) and medial motor column (MMC) neurons (Agalliu et al., 2009; Jung et al., 2010). By contrast, upon Hoxc9 activity at thoracic level, MN diversify into three columns called MMC, hypaxial motor column (HMC) and visceral preganglionic column (PGC) (Agalliu et al., 2009; Dasen et al., 2008; Dasen and Jessell, 2009b; Dasen et al., 2003; Jung et al., 2010). Similar to brachial level, Hoxd10 and Hoxc10 contribute to the specification of lumbar MNs into LMC and MMC neurons (Dasen and Jessell, 2009b; Dasen et al., 2003; Dasen et al., 2005). Interestingly, Hox cofactors such as Meis, Pbx or Foxp1 refine and constrain Hox activity within distinct MN subclasses. Foxp1, whose expression is selectively induced by Hox6/10 and Hoxc9 in LMC and PGC neurons, respectively, acts jointly with Hox transcription factors to specify LMC or PGC fate (Dasen et al., 2008; Jung et al., 2010; Rousso et al., 2008). In addition, Foxp1 and Hox proteins synergistically control motor axon projections and axon targeting of LMC and of PGC neurons (Dasen et al., 2008; Rousso et al., 2008). Finally, Hox genes regulate the organization into specific motor pools (Dalla Torre di Sanguinetto et al., 2008; Dasen and Jessell, 2009b; Dasen et al., 2005).

Multiple distinct IN cell types are present in the adult spinal cord (Jankowska, 2001b, 2008; Johnson and Sears, 1988; McDonagh et al., 1998, 1999) (for review (Goulding, 2009; Grillner and Jessell, 2009)). Many of which are thought to arise from the V0, V1, V2 and V3 cardinal classes, although dorsal embryonic INs are also likely to contribute to the motor circuits (for review (Arber, 2012; Garcia-Campmany et al., 2010; Lupo et al., 2006)). These ventral IN populations have been primarily characterized at brachial or at lumbar levels of the developing spinal cord (Litington and Chiang, 2000; Parras et al., 2002), and it is now

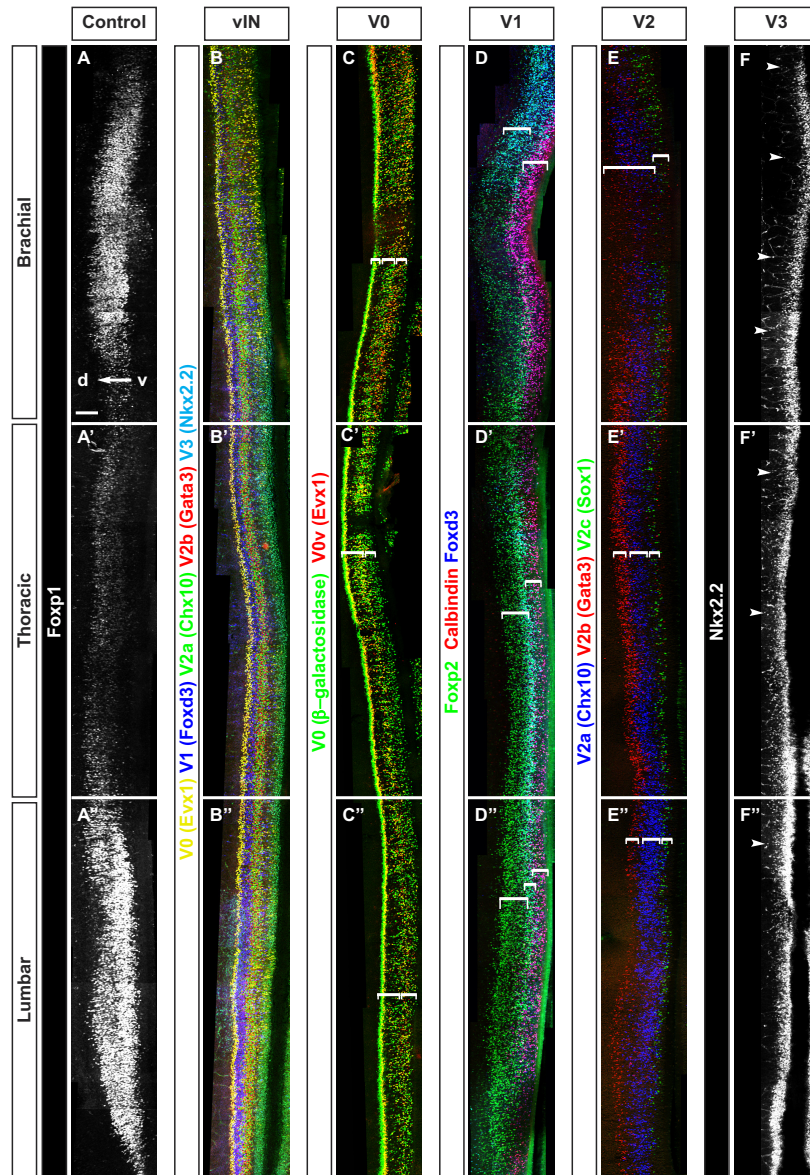
apparent that these populations diversify into several subpopulations. Indeed, V0 INs subdivide into a dorsal (V0<sub>D</sub>), a ventral (V0<sub>V</sub>), a cholinergic (V0<sub>C</sub>) and a glutamatergic (V0<sub>G</sub>) complement (Zagoraïou et al., 2009). V0<sub>D</sub> are inhibitory commissural INs that control left/right alternation (Lanuza et al., 2004). V1 INs sequentially differentiate into several inhibitory cell types including Renshaw cells (RCs), Ia INs and other unidentified subpopulations (Alvarez et al., 2005; Benito-Gonzalez and Alvarez, 2012; Gosgnach et al., 2006; Siembab et al., 2010; Stam et al., 2012). In contrast, V2 INs divide equally into V2a excitatory and V2b inhibitory INs (Karunaratne et al., 2002; Kimura et al., 2008; Lee et al., 2008; Lundfald et al., 2007; Peng et al., 2007), and in a small subpopulation of V2c INs (Panayi et al., 2010). While V2a INs regulate burst robustness and left/right coordination during walking, the physiological functions of V2b and V2c cells remain to be identified. Finally, a majority of V3 neurons are excitatory INs that segregate into a ventral (V3<sub>V</sub>) and a dorsal (V3<sub>D</sub>) subpopulation, which additionally differ in their electrophysiological properties (Grossmann et al., 2010a; Zhang et al., 2008). V3 neurons constitute a population of commissural INs that ensure a regular and balanced motor rhythm during walking by distributing excitatory drive towards both halves of the spinal cord (Zhang et al., 2008).

Despite an increased understanding of the developmental programs that specify these cardinal ventral IN classes, very little is known about their topographic distribution and organization along the rostrocaudal axis of the spinal cord. The diversification of these cardinal classes of ventral INs into distinct subsets and the relationship between these subsets and adult IN populations also deserves further investigation. A major factor preventing progress is the incomplete characterization of molecular or genetic markers that define V0<sub>D</sub>, non-RCs V1 or V3<sub>V</sub>/V3<sub>D</sub> subpopulations. While recent studies have described the expression of several transcriptional regulators in the developing spinal cord, including BhlhB5

(Skaggs et al., 2011) or *Prdm8* (Komai et al., 2009), the Cut homeobox transcription factors of Onecut family such as HNF-6, OC-2 and OC-3 (Francius and Clotman, 2010) and the bZIP transcription factors MafA, MafB (Stam et al., 2012) and cMaf, the relationship of these molecular markers to ventral IN subdivisions and known cell types has not been analyzed comprehensively.

Here, we characterized the topographic distribution of ventral INs along the rostrocaudal axis of the spinal cord using immunofluorescence labeling in combination with 3D imaging on whole mounted spinal cords. Our findings revealed pronounced differences in the distribution of ventral INs at brachial, thoracic and lumbar levels. We found that a majority of  $V0_D$  neurons contain *BhlhB5* whereas *Olig3* appears to define a majority of  $V3_V$  neurons. We also observed that each cardinal class of ventral INs can be subdivided into several subsets according to combinatorial expression of multiple transcription factors. Taken together, these findings provide evidence that the cardinal classes of ventral INs are differentially distributed along the rostrocaudal axis of the spinal cord and subdivide into different subsets characterized by distinct combinations of molecular markers.





**Figure 23: Ventral spinal interneurons are differentially distributed along the rostrocaudal axis of the spinal cord.** Whole mount immunofluorescence labeling of Fxp1 or ventral interneuron markers on whole mount spinal cord of mouse embryo at e12.5. (A-A'') Fxp1 (white) shown as reference to delineate the brachial, thoracic and lumbar regions of the developing spinal cord. (B-B'') Differential distribution of ventral spinal interneurons including V0<sub>v</sub> (yellow), V1 (blue), V2a (green), V2b (red) and V3 (cyan) at brachial (B), thoracic (B') or lumbar (B'') levels of the spinal cord. (C-C'') V0 interneurons including V0<sub>v</sub> (red or yellow) and V0<sub>d</sub> (green) display distinct distribution pattern at brachial (C), thoracic (C') or lumbar (C'') levels in *Dbx1*<sup>lacZ/+</sup> embryos. (D-D'') V1 interneurons including Renshaw cells (red or magenta) and V1 non-Renshaw cells that contain Foxp2 (green) or →

### **3.1.3. Results**

#### **Ventral spinal INs are differentially distributed along the rostrocaudal axis of the spinal cord.**

Different classes of cardinal INs have been described in the developing ventral spinal cord (Jovanovic and Burke, 2004; Litingtung and Chiang, 2000; Morikawa et al., 2009b; Parras et al., 2002; Schotland and Tresch, 1997). However, most of these populations have been characterized at a single rostrocaudal level, and the respective distribution of these embryonic neuronal populations along the rostrocaudal axis of the spinal cord has not been extensively described. To address this question, we first examined the topographic distribution of the ventral INs at e12.5 by immunofluorescence on whole mount spinal cord combined with 3D imaging. At this developmental stage, the ventral spinal INs have been generated and a majority of them have reached their final position. Furthermore, the ventral spinal cord is still devoid of glial cells (Agius et al., 2004; Hochstim et al., 2008; Muroyama et al., 2005) and ventral INs have not yet undergone developmental cell death (Prasad et al., 2008), which, at a later stage, would complicate the analyses. Multiple labeling of each cardinal interneuron population revealed that these cells are not identically distributed along the rostrocaudal axis of the spinal cord (Figure 23B-B"). Indeed, at thoracic levels, a ventral to dorsal (Figure 23A) series of columns corresponding to V3, sparse V1,

---

Foxd3 (blue) are differentially distributed at brachial (D), thoracic (D') or lumbar (D'') along the rostrocaudal axis. (E-E'') V2 interneurons including V2a (blue), V2b (red) and V2c (green) exhibit distinct distribution pattern at brachial (E), thoracic (E') or lumbar (E'') levels. (F-F'') By contrast, V3 interneurons (white) display relative homogenous distribution, along the rostrocaudal axis of the spinal cord. The white brackets indicate distinct columnar organization within V0, V1 and V2 neurons while white arrowheads show few cells distributed outside V3 neuron column. The white arrow indicates the ventral (v) to dorsal (d) axis. Scale bar = 100µm.

intermingled V2a, V2b and V3 and finally V0<sub>v</sub> INs were observed (Figure 23B'). At variance, the organization, the size and the relative position of these columns seemed different at brachial compared to lumbar levels (Figure 23B-B"). To gain further insights into these possible differential distributions, each cardinal class of ventral INs was analyzed separately.

The V0 INs were visualized in Dbx1<sup>LacZ/+</sup> mouse embryos, in which b-galactosidase is detected in V0 progenitors and in a majority of differentiating V0 cells (Pierani et al., 2001), but not in V1 or in dorsal dl6 INs (Fig.S5A-F"). Evx1 was detected as a specific marker of V0<sub>v</sub> INs (Moran-Rivard et al., 2001; Pierani et al., 2001). At brachial levels, the V0 neurons were organized into 3 columns (brackets in Figure 23C, Fig.S1A, and Movie 1A). The dorsal column corresponded to the V0 progenitors and newly-born V0<sub>v</sub> (Evx1<sup>+</sup>) or V0<sub>d</sub> neurons. The intermediate column contained a large group of V0<sub>d</sub> intermingled with some V0<sub>v</sub> cells. The ventral column was composed of a large group of V0<sub>v</sub> intermingled with fewer V0<sub>d</sub> INs. By contrast, at thoracic levels, the intermediate column included a large portion of V0<sub>v</sub> and fewer V0<sub>d</sub>, while the ventral column was composed almost exclusively of V0<sub>d</sub> cells (brackets in Figure 23C', Fig.S1B, Movie 1B). At lumbar levels, we observed two major columns distributed between a dorsal complement of tightly packed V0 progenitors and newly-born V0<sub>d</sub> or V0<sub>v</sub> cells, and a ventral complement with intermingled V0<sub>d</sub> and V0<sub>v</sub> neurons (brackets in Figure 23C", Fig.S1C, Movie 1C). Note that a small fraction of V0 INs displayed low levels of b-galactosidase at brachial levels, probably due to b-galactosidase degradation in the earliest-born cells. Furthermore, the distribution of V0 INs (and of the other cardinal populations, see below) at brachial or lumbar levels varied according to the differential distribution of the MNs in these regions, as previously reported (Alvarez et al., 2005). Thus, V0 INs exhibited a differential distribution of the V0<sub>d</sub>

and V0<sub>v</sub> subpopulations, including changes in their relative dorso-ventral position, along the rostrocaudal axis of the spinal cord.

V1 INs divide into RCs (Foxd3<sup>+</sup>/Calbindin<sup>+</sup>) and V1 non-RC INs (Foxp2<sup>+</sup>/Foxd3<sup>+</sup> or Foxd3<sup>+</sup>) that include inhibitory Ia INs and other uncharacterized populations (Gosgnach et al., 2006; Morikawa et al., 2009a; Siembab et al., 2010; Stam et al., 2012). At brachial levels, V1 neurons were distributed in a ventral column that contained only RCs and a large dorsal column composed of numerous Foxp2<sup>+</sup> V1 neurons and rare Foxd3<sup>+</sup> V1 cells (brackets in Figure 23D, Fig.S2A, and Movie 2A). At thoracic levels, the V1 gathered in a less-extended dorso-ventral portion of the spinal cord (brackets in Figure 23D') with a progressive reduction in RCs from rostral to caudal, and Foxp2<sup>+</sup>/Foxd3<sup>+</sup> cells grouped in a central column (brackets in Figure 23D', Fig.S2B, Movie 2B). At lumbar levels, V1 INs were slightly more numerous than at thoracic levels. However, fewer RCs were observed and were spread more dorsally while Foxd3 levels decreased along the cranial to caudal axis (Figure 23D'', Fig.S2C, Movie 2C). Thus, the amount and the divisions of V1 neurons varied between limb and thoracic levels.

V2 INs segregate into V2a (Chx10<sup>+</sup>), V2b (Gata3<sup>+</sup>) and V2c (Sox1<sup>+</sup>) subpopulations (Panayi et al., 2010; Peng et al., 2007; Zhou et al., 2000). At brachial levels, V2b neurons were widely distributed from very ventral to medial regions, whereas V2a intermingled with V2b neurons in a central column and V2c gathered close to the ventral most V2b cells (brackets in Figure 23E, Fig.S3A, and Movie 3A). At thoracic levels, V2b INs were more dorsally restricted, being almost absent from ventral regions, whereas V2a gathered in a narrower central column directly ventral to the V2b cells (brackets in Figure 23E', Fig.S3B, Movie 3B). At lumbar levels, V2a INs were more numerous and expanded along the dorso-ventral axis. V2b cells were less numerous in the rostral portion but more numerous and more extended along the dorso-ventral axis in the caudal portion of the lumbar region. V2c were

less numerous with respect to V2a and V2b (brackets in Figure 23E", Fig.S3C, Movie 3C). Thus, the V2 INs presented a differential distribution along the rostrocaudal axis of the spinal cord.

Finally, V3 INs labeled for their specific marker Nkx2.2 were organized in a single ventral column homogenous along the rostrocaudal axis of the spinal cord, although a few of them were distributed dorsally more frequently within the brachial and thoracic levels, but less frequently in the lumbar region (arrowheads in Figure 23F-F", arrowheads in Fig.S4A-C, Movie 4A-C).

Together, these observations provided evidence that the cardinal classes of ventral INs are differentially distributed along the rostrocaudal axis of the spinal cord except for V3, which exhibited a more homogenous distribution pattern.

### **Ventral spinal INs can be subdivided into subsets with distinct molecular identity**

The four cardinal classes of ventral INs give rise to dozens of distinct neuronal populations in the adult, suggesting that they subdivide into numerous subsets. Differential location of these subsets may contribute to the differential distribution reported above. In an effort to address this question and to identify molecular markers of subsets of embryonic ventral INs, we analyzed the distribution of a set of transcription factors that have been described in the ventral part of the developing spinal cord relative to established markers of ventral IN populations. The transcription factor set included BhlhB5 (also known as Bhlhe22)(Brunelli et al., 2003; Liu et al., 2007; Skaggs et al., 2011), Prdm8 (Komai et al., 2009; Ross et al., 2012), HNF-6, OC-2, OC-3 (Francius and Clotman, 2010), Nurr1 (Li et al., 2011), Pax2 (Burrill et al., 1997), Pax6, MafA, MafB or cMaf

(Hu et al., 2012), Pitx2 (Zagoraiou et al., 2009), Olig3 (Takebayashi et al., 2002), Pou3F1 and Pou4F1 (Fedtsova and Turner, 1995).

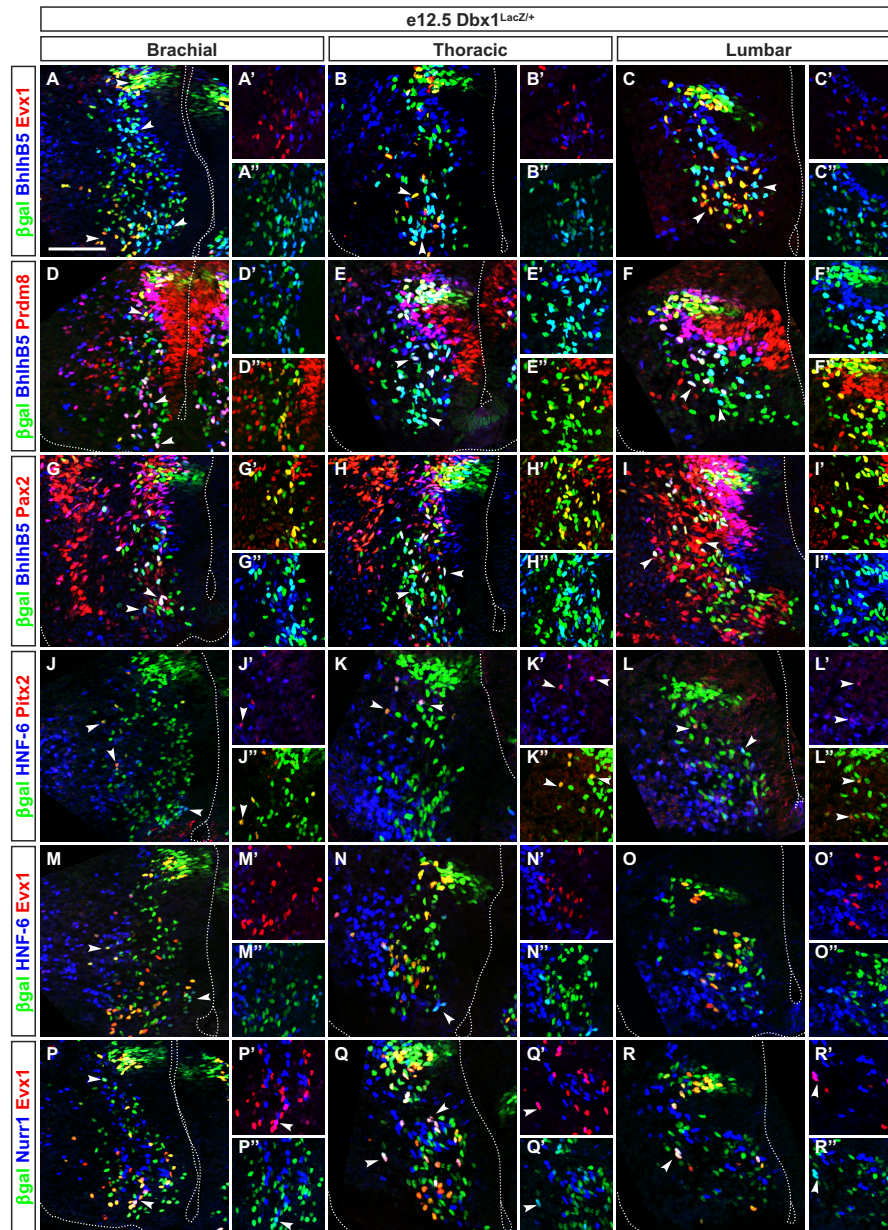
### **Homogenous subsets of V0 INs along the rostrocaudal axis of the spinal cord**

Previous studies established that V0 INs diversify into distinct subpopulations including V0<sub>D</sub>, V0<sub>V</sub> (Evx1<sup>+</sup>), V0<sub>C</sub> (Evx1<sup>+</sup>/Pitx2<sup>+</sup>/ACh<sup>+</sup>) and V0<sub>G</sub> (Evx1<sup>+</sup>/Pitx2<sup>+</sup>) neurons (Lanuza et al., 2004; Pierani et al., 2001; Zagoraiou et al., 2009). Using Dbx1<sup>LacZ/+</sup> embryos, we determined whether additional candidate markers were present in V0 INs. Surprisingly, we found that BhlhB5, a transcriptional repressor expressed in dorsal dl6 INs, V1 and V2a INs (Brunelli et al., 2003; Liu et al., 2007; Skaggs et al., 2011) was additionally detected in 77% to 89% of Evx1<sup>+</sup> V0 INs, namely V0<sub>D</sub> (Figure 24A-C"; Fig.S5A-D"; Figure 29A-C). In contrast, BhlhB5 was not present in Evx1<sup>+</sup> V0<sub>V</sub> INs (Figure 24A-C"), as described previously (Skaggs et al., 2011). Hence, BhlhB5 is a marker for a majority of V0<sub>D</sub> neurons. This observation also highlights further diversification of V0<sub>D</sub> INs into BhlhB5<sup>+</sup> or BhlhB5<sup>-</sup> cells.

At brachial levels, the BhlhB5<sup>+</sup> V0<sub>D</sub> INs accounted for 50% of the V0 INs (Figure 29A) and were located close to the central canal. About half of these cells contained the PR Domain Zinc Finger Protein Prdm8, a cofactor of BhlhB5 (Figure 24D-D", Figure 29B) or Pax2 (Figure 24G-G", Figure 29C). Smaller subsets were labeled for Onecut factors (arrowheads in Figure 24J-J", M-M"; Figure 29D), Pax6 or the orphan nuclear receptor Nurr1 (Nr4a2), a protein previously detected in glutamatergic cortical neurons and dopaminergic neurons (arrowheads in Figure 24P-P"; Figure 29A, E; data not shown) (Andjelic et al., 2009; Arimatsu et al., 2003). V0<sub>V</sub> accounted for 40% of the V0 INs (Figure 29A). They migrated ventro-medially to become located lateral to BhlhB5<sup>+</sup> V0<sub>D</sub> INs (Figure 24A-A"). They

divided into major subsets labeled for OC-2 or OC-3 (Fig.S6A-B) and Nurr1 (Figure 24P-P", Figure 29E) and into minor subsets containing Prdm8<sup>+</sup> (Figure 24D-D", Figure 29B), HNF-6<sup>+</sup> (Figure 24J-J", Figure 29D) and Pax2<sup>+</sup> (Figure 24M-M", Figure 29C). Finally, V0<sub>C</sub> and V0<sub>G</sub> INs defined by Pitx2 formed a small subset of V0<sub>V</sub> (Figure 24G-G") and a majority of them contained HNF-6 (arrowheads in Figure 24J-J", Figure 29D). At thoracic and lumbar levels, the proportion of V0<sub>D</sub> and V0<sub>V</sub> did not significantly change compared to brachial levels (Figure 24B-B", Figure 29A). Likewise, the proportions of V0 subsets remained relatively stable, with the exception of an increase in HNF-6<sup>+</sup> and in OC-3<sup>+</sup> V0<sub>V</sub> that correlated with a decrease in HNF-6<sup>+</sup> and in OC-3<sup>+</sup> V0<sub>D</sub> INs at thoracic levels of the spinal cord (Figure 29A-E, Fig.S6A-B).

Thus V0<sub>V</sub> and V0<sub>D</sub> proportion did not significantly change along the rostrocaudal axis of the spinal cord, and each V0 subpopulation can be divided into several subsets according to a combinatorial code that includes Pax2, Prdm8, Pax6, Nurr1 or Onecut factors, which are differentially distributed between brachial, thoracic and lumbar levels.



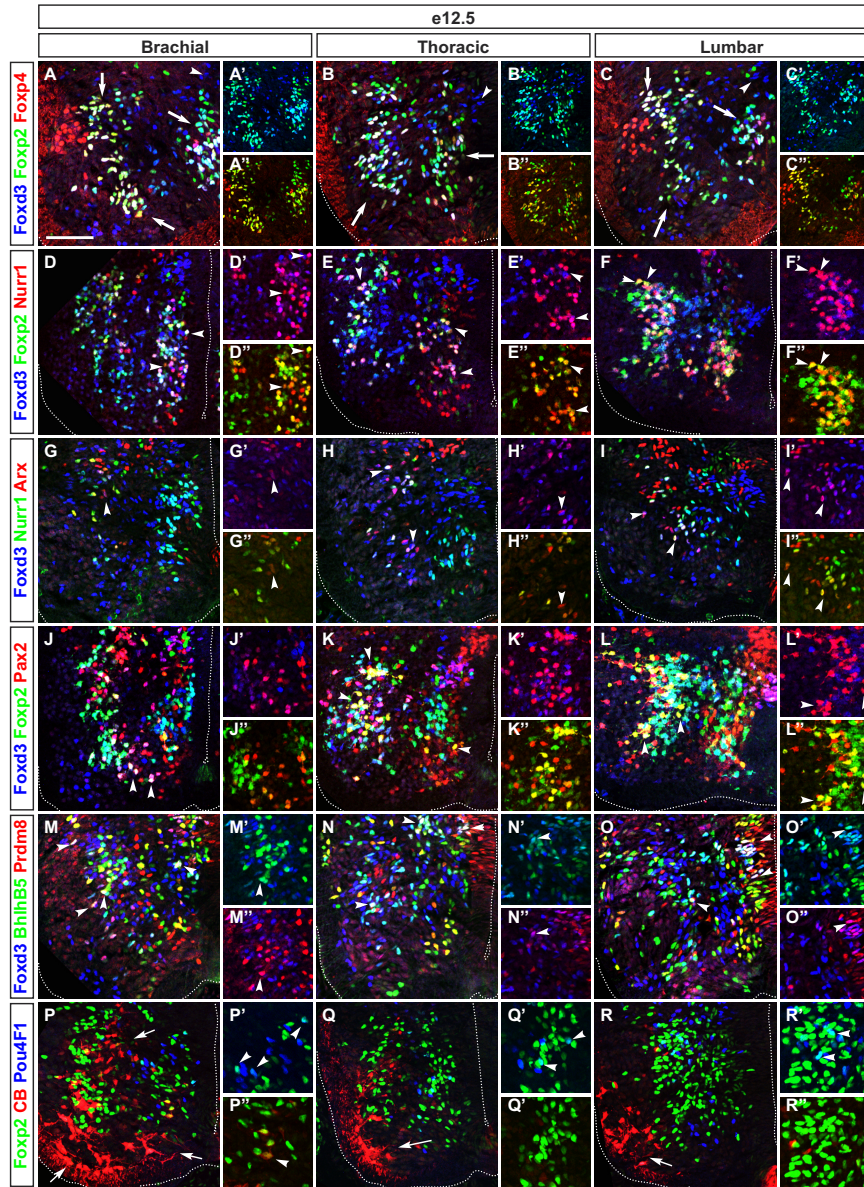
**Figure 24: Homogenous distribution of V0 interneuron subsets along the rostrocaudal axis of the spinal cord.** (A-A'') Immunofluorescence labeling on transverse section at brachial levels of the spinal cord of  $Dbx1^{LacZ/+}$  embryo at e12.5.  $V0_D$  interneurons contain BhlhB5 (cyan) are intermingled with  $Evx1^+$   $V0_V$  neurons (yellow or red). Most of  $V0_V$  cells are located ventrally as indicated by left arrowhead while  $V0_D$  are homogenously distributed between the vicinity of the V0 progenitor domain and the ventro-medial region of the spinal cord as shown by right arrowhead. →



## V1 interneuron subsets are differentially distributed along the rostrocaudal axis of the developing spinal cord

V1 INs characterized by *Foxd3* and *En1* expression arise from the p1 progenitor domain defined by combined expression of *Dbx2*, *Nkx6.2* and *Pax6* (Matise and Joyner, 1997; Pierani et al., 1999; Pierani et al., 2001; Sapir et al., 2004). Neurons of this class rapidly diversify into several inhibitory interneuron subpopulations including RCs characterized by the presence of calbindin and *Foxd3*, and V1 non-RCs containing Ia INs and other V1 subpopulations characterized by *Foxp2*, *Foxd3*,

(B-B'') At thoracic levels,  $V0_V$  (yellow or red) amounted less in the ventral territory of V0 interneurons compared to brachial levels (see arrowhead). Conversely, most of  $V0_D$  in magenta are gathered in this ventral territory as indicated by arrowhead. (C-C'') At lumbar levels,  $V0_V$  (yellow or red) form a relatively homogenous group located in the ventral territory (left arrowhead), while  $V0_D$  (cyan) distribute among dorsal, ventro-medial and ventro-lateral territories (right arrowhead). (D-D'') At brachial levels, *Prdm8* is detected V0 progenitors and in subsets of  $V0_V$  (yellow) located close to the central canal (arrowheads). By contrast, *Prdm8* is present in a few number of  $V0_D$  cells (white) as shown by bottom arrowhead. (E-E'') At thoracic levels,  $Prdm8^+$   $V0_V$  interneurons (yellow) are located close to progenitor domain and in a dorso-ventral direction, while the amount of  $Prdm8^+$   $V0_D$  (white) is increased (see arrowheads). (F-F'') At lumbar levels, subsets of  $V0_D$  that are  $Prdm8^+$  (white) are located in a dorso-ventral direction between the V0 progenitor domain and  $V0_V$  neurons (yellow or green) as indicated by arrowheads. (G-G'') At brachial levels, *Pax2* is present in a large subset of  $V0_D$  neurons (white) and in a small subset of  $V0_V$  neurons (yellow) distributed between the V0 progenitor domain and  $V0_V$  ventral territory (see arrowheads). (H-H'') The  $Pax2^+$  V0 subsets are similarly distributed at thoracic and lumbar levels. (J-J'') At brachial levels, *Pitx2* is expressed in  $V0_C$  interneurons (yellow) which are located laterally to the V0 interneuron migration flux as indicated by arrowheads. A portion of  $V0_C$  that co-expresses HNF-6 (magenta) is present ventrally (see bottom arrowhead). (K-K'') At thoracic levels, most of  $V0_C$  express HNF-6 (magenta).  $V0_C$  are detected laterally to the V0 progenitor domain and dorsally to  $V0_V$  ventral territory as indicated by arrowheads. (L-L'') At lumbar levels, the amount of  $V0_C$  (yellow) is reduced compared to brachial or thoracic levels (see left arrowhead). (M-M'') At brachial levels, HNF-6 is detected in a small subset of  $Evx1^+$   $V0_V$  interneurons (white or magenta) as indicated by left arrowhead and in a subset of  $V0_D$  (cyan) indicated by right arrowhead. (N-N'') At thoracic levels, HNF-6 is restricted to a few number of  $V0_V$  (white) and of  $V0_D$  (cyan) indicated by arrowhead. (O-O'') At lumbar levels, HNF-6 is present in very few  $Evx1^+$  cells (white or magenta) or  $V0_D$  (cyan) neurons compared to brachial or thoracic regions. (P-P'') At brachial levels, *Nurr1* expression is detected in a small subset of V0 including  $V0_V$  (white or magenta) indicated by bottom arrowhead and  $V0_D$  (cyan) located ventrally, close to the floor plate (see arrowhead in insets). (Q-Q'') At thoracic levels,  $Nurr1^+$  subsets of  $V0_V$  (white or magenta) indicated by arrowheads or of  $V0_D$  (cyan) are expanded. (R-R'') By contrast, at lumbar levels, *Nurr1* expression is only detected in small subsets within  $V0_V$  (white or magenta) indicated by arrowhead and  $V0_D$  (cyan). Scale bar = 100 $\mu$ m.



**Figure 25: Differential distribution of V1 interneuron subdivisions along the rostrocaudal axis of the spinal cord.** (A-A'') Immunofluorescence labeling on transverse section at brachial levels of embryonic spinal cord at e12.5 shows that V1 interneurons are subdivided into 3 large subsets (arrows) containing Foxp2 (cyan). Foxp4 is detected in a portion of these Foxp2<sup>+</sup> V1 neurons (white). (B-B'') At thoracic levels, Foxd3<sup>+</sup> V1 neurons form 2 groups (arrows) that contain Foxp2 (in green/cyan) and Foxp4 (in red/white). (C-C'') At lumbar levels, V1 interneurons distribute among 3 groups (arrows) and the ratio between Foxp2<sup>+</sup> neurons and Foxp4<sup>+</sup>/Foxd3<sup>+</sup> cells decreased compared to brachial or →

and En1 (Benito-Gonzalez and Alvarez, 2012; Gosgnach et al., 2006; Matisse and Joyner, 1997; Morikawa et al., 2009a; Stam et al., 2012). Noticeably, Foxd3 and Foxp1 are also present in dl2 INs, but these cells are not yet located in the ventral spinal cord at e12.5 (Gross et al., 2002; Muller et al., 2005).

At brachial levels, non-RC V1 amounted for 70% of the V1 INs. A large non-RC subset contained Foxp2, partly in combination with Foxp4 and/or Foxd3 (Figure 25A-A", Figure 29F). This large subset spatially divided into 3 groups, i.e. a medial, a ventro-lateral and a lateral subdivision (arrows in Figure 25A-A"). In addition, we detected a subset of non-RC V1 that contained Foxd3 but not Foxp2 nor Foxp4 in the vicinity of the p1 progenitor domain (arrowhead in Figure 25A-A", Figure 29F).

thoracic levels. (D-D") At brachial levels, Nurr1 is detected in subsets (arrowheads) within 2 large V1 groups containing Foxp2 (yellow). (E-E") At thoracic levels, Nurr1<sup>+</sup> subsets (white) are intermingled with Foxp2<sup>+</sup> group (cyan and green) close to the central canal and/or (in/with Foxp2<sup>+</sup> group close) to motor neurons as indicated by arrowheads. (F-F") At lumbar levels, Nurr1<sup>+</sup> subsets are included in 2 groups of V1 that contain Foxp2 (yellow) indicated by arrowheads or Foxd3 and Foxp2 (white). (G-G") At brachial levels, Arx is present in a few number of Foxd3<sup>+</sup> V1 neurons (magenta) as indicated by arrowhead. (H-I") By contrast, at thoracic and lumbar levels, Arx is observed in subsets of Foxd3<sup>+</sup> (magenta) and of Foxd3<sup>+</sup>/Nurr1<sup>+</sup> (white) V1 neurons (arrowheads). (J-J") At brachial levels, Pax2 expression defines subsets in Foxd3<sup>+</sup> (magenta) and in Foxp2<sup>+</sup>/Foxd3<sup>+</sup> (white) V1 interneurons (arrowheads). (K-K") At thoracic levels, Pax2 is distributed in Foxd3<sup>+</sup> (magenta), Foxd3<sup>+</sup>/Foxp2<sup>+</sup> (white) and Foxp2<sup>+</sup> subsets (yellow) as indicated by arrowheads. (L-L") At lumbar levels, Pax2<sup>+</sup> subsets (yellow or white) are located within V1 containing Foxp2 located medially or close to motor neurons (arrowheads). (M-M") At brachial levels, BhlhB5 is detected in subsets of Foxd3<sup>+</sup> V1 neurons (cyan) located close to the central canal or to motor neurons. Prdm8 is present in small subsets of V1 neurons located close to V1 progenitor domain or more ventrally, close to motor neurons. A small subset of V1 that co-express BhlhB5 and Prdm8 (white) is observed in the vicinity of the motor neurons (arrowheads). (N-N") At thoracic levels, the amount of V1 neurons that contain BhlhB5 (cyan), or BhlhB5 and Prdm8 (white) indicated by arrowheads, is increased while a few number of Prdm8<sup>+</sup> V1 cells is detected. (O-O") At lumbar levels, the amount of cells corresponding to Prdm8<sup>+</sup> (magenta) or BhlhB5<sup>+</sup> V1 neurons (cyan) decreases while BhlhB5<sup>+</sup>/Prdm8<sup>+</sup> co-expressing V1 (white) indicated by arrowheads, increase compared to the thoracic region. (P-P") At brachial levels, Pou4F1 is present in a few Foxp2<sup>+</sup> V1 neurons (cyan) indicated by arrowheads. Renshaw cells (red) are distributed in 3 groups (arrows) with very few containing Foxp2 (yellow) indicated by arrowhead. (Q-Q") At thoracic levels, the amount of Renshaw cells (red) decreases (arrow) while Foxp2<sup>+</sup> V1 expressing Pou4F1 (cyan) slightly increases (arrowheads). (R-R") At lumbar levels, similar to thoracic levels, the amount of Renshaw cells (red) decreases (arrow) but Pou4F1<sup>+</sup> subset (cyan) is detected dorsally, close to motor neurons (arrowheads). Scale bar = 100µm.

The Foxp2<sup>+</sup> V1 subset could be further subdivided based on the distribution of Nurr1. This protein was detected in a small subset of V1 that contained Foxp2 but not Foxd3 located in the dorsal part of the lateral subdivision of V1 and in a large subset that contained Foxp2 and Foxd3 located in the medial subdivision (arrowheads in Figure 25D-D", Figure 29G). Similarly, Pax2 was detected in the ventro-lateral subdivision that contained Foxp2 and Foxd3 (arrowheads in Figure 25J-J", Figure 29H). At variance, Foxp1 was present in the medial subdivision of non-RC V1 INs and in the lateral subdivision that contained Foxd3, close to MNs (arrowheads in Fig.S5G-I", Figure 29J). Arx was detected in a small subset of the lateral group of Foxp2<sup>+</sup>/Nurr1<sup>+</sup> V1 INs (arrowheads in Figure 25G-G", Fig.S6C), BhlhB5 and Prdm8 were partly codetected in a subset of V1 INs located close to the p1 domain that corresponds to newly-born V1 INs and in the lateral complement, close to the MNs (arrowheads in Figure 25M-M", Fig.S6D). Finally, Pou3F1 (also known as Scip or Oct6) and Pou4F1 (also known as Brn3a) were detected in a very limited number of V1 INs (arrowheads in Figure 25P-P", Figure 29I-J, Fig.S5G-G"). On the other hand, RCs amounted for 30% of V1 cells (Figure 29I). They divided into 3 distinct groups, i.e. a ventro-medial subdivision close to the lumen of the spinal cord, a ventro-lateral subdivision that bordered the MNs and a third group located dorsally to this ventro-lateral subdivision (arrows in Figure 25P-P"). Foxp2 was detected in a few of these cells (arrowhead in Figure 25P", Figure 29I).

At thoracic and lumbar levels, Foxp2<sup>+</sup> non-RC V1 INs organized in a lateral and a medial subdivision (arrows in Figure 25B-B", Figure 29F). Among these, the respective amount of each subset was relatively stable (Figure 29F-J). However, V1 neurons containing exclusively Foxd3 were more numerous at thoracic levels whereas those containing exclusively Foxp2 decreased at lumbar levels, and V1 subsets characterized by the presence of Arx or Nurr1 were more abundant at

thoracic or at lumbar levels, respectively (arrowheads in Fig25D-I", Figure 29G, Fig.S6C). In contrast, the Pax2<sup>+</sup> subset was decreased at thoracic levels (Figure 25J-L", Figure 29H), whereas the Bhlhb5<sup>+</sup> one was increased (Figure 25M-O", Fig.S6D). RCs were progressively less numerous in more caudal regions, as reported previously (Alvarez et al., 1999; Benito-Gonzalez and Alvarez, 2012; Ryall et al., 1971; Stam et al., 2012). In addition, they gathered in a unique group located laterally, close to the basal lamina and the MNs (Figure 25Q-Q", Figure 29I).

In summary, V1 INs were distributed into RCs and into 3 or 2 large subpopulations of Foxp2<sup>+</sup> or Foxd3<sup>+</sup> cells. Each V1 subpopulation except for RCs can be subdivided in several subsets distinguished by the single or the combinatorial expression of Pax2, Nurr1, Pou4F1, Bhlhb5 or Prdm8, the distribution and the amount of which differ according to the rostrocaudal level.

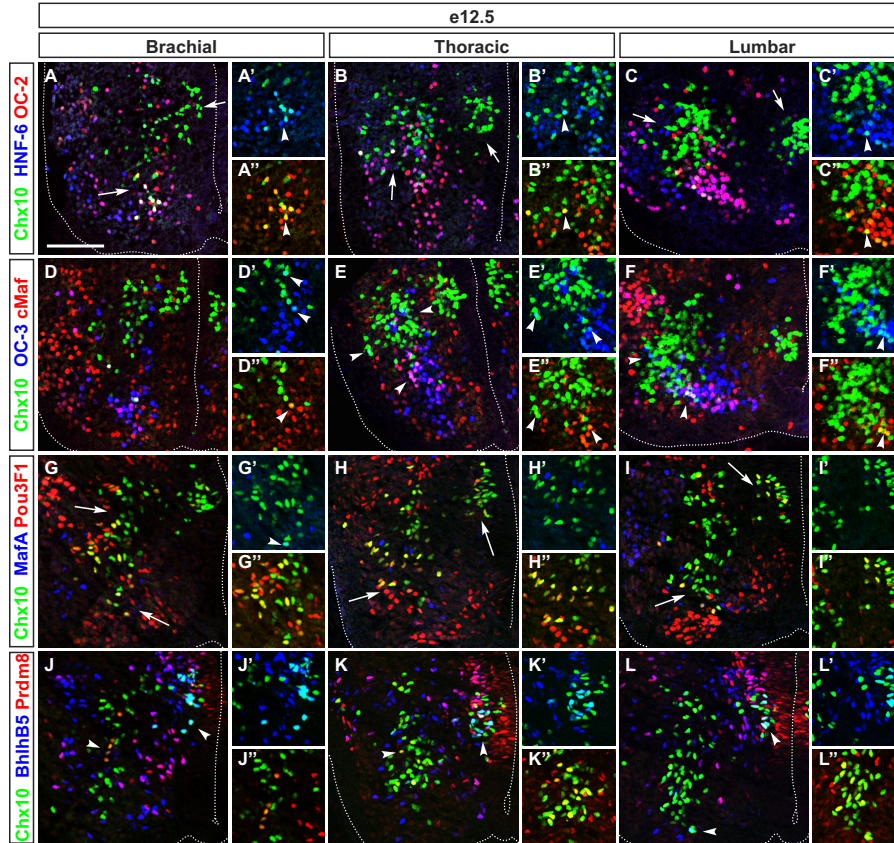
## **V2 interneuron subsets are homogeneous along the rostrocaudal axis of the spinal cord**

V2 INs are produced from the p2 domain defined by combined expression of Foxn4, Mash1 (rename Ascl1), Lhx3 and Gata2 (Joshi et al., 2009; Peng et al., 2007; Smith et al., 2002). They are distributed between the excitatory V2a subpopulation defined by Chx10 expression and the V2b and V2c inhibitory subpopulations characterized by the presence of Gata2/Gata3 and Sox1, respectively (Panayi et al., 2010).

At brachial levels of the spinal cord, V2a INs segregated into a medial and a ventral subdivision (arrows in Figure 26A-A"). The three members of the *Onecut* family were present in a subset of the ventral subdivision in a partially overlapping pattern (arrowheads in Figure 26A-A", D-D", Figure 29K-L). Conversely, Pou3F1 was detected in a large subset of V2a located laterally and ventrally (arrows in



Figure 26G-G", Figure 29M). Prdm8, Bhlhb5, cMaf and MafA further defined subsets of the lateral V2a neurons (Figure 26D-D", 26G-G", 26J-J", Figure 29L-N).



**Figure 26: V2a interneurons subdivisions are differentially distributed along the rostrocaudal axis of the spinal cord.** (A-A'') Immunofluorescence labeling on transverse section at brachial levels of e12.5 embryonic spinal cord shows that V2a interneurons subdivide into medial and lateral groups (arrows). Lateral group comprise small ventral subsets (arrowhead) that contain HNF-6 (cyan), OC-2 (yellow) or HNF-6 and OC-2 (white). (B-B'') At thoracic levels, OC-2<sup>+</sup> subset (yellow) is absent, but HNF-6<sup>+</sup>/OC-2<sup>+</sup> subset (white) is detected dorsally to motor neurons (arrowhead) while the HNF-6<sup>+</sup> subset (cyan) is expanded in lateral group (left arrow). (C-C'') At lumbar levels, the amount of HNF-6<sup>+</sup>/OC-2<sup>+</sup> (white) indicated by arrowhead or of HNF-6<sup>+</sup> V2a neurons (cyan) decreases at lateral group (left arrow). (D-D'') At brachial levels, a ventral subset of V2a contains cMaf and OC-3 (white), while OC-3 is present in subsets of V2a (cyan) located laterally to newly-born V2a interneurons (arrowheads). (E-E'') At thoracic levels, V2a that contain OC-3 (in cyan) are less numerous while cMaf<sup>+</sup>/OC-3<sup>+</sup> subset (white) located ventrally is still present (arrowheads). (F-F'') At lumbar levels, the amount of V2a containing OC-3 (cyan) is increased (left arrowhead) and this subset is located dorsally to the cMaf<sup>+</sup>/OC-3<sup>+</sup> division (white) as indicated by bottom arrowheads. (G-G'') At brachial levels, Pou3F1 defines a large subset of V2a (yellow) located laterally and another one close to the newly-born V2a→

## Results

To characterize V2b subsets, we used  $Gata3^{Cre/+};Rosa26^{floxstop-tdTomato}$  mouse embryos in which V2b and V2c are permanently labeled by the expression of tdTomato. V2b migrated laterally from their progenitor domain toward the MNs of the LMC, then turned toward a dorsal or a ventral direction to medially surround the MNs (Figure 27A). Three V2b subsets were identified based on their distribution, on the levels of Gata2 and Gata3, and on their location along the dorso-ventral axis (Figure 27A-A"). Gata2 and Gata3 were codetected in a large subpopulation of V2b, located medially to the MNs (white arrow in Figure 27A-A", Figure 29O). Gata2 alone was detected in a small subpopulation of newly-born V2b close to the progenitor domain (blue arrow in Figure 27A-A") whereas a dorso-lateral subset of V2b was defined by a high levels of Gata3 ( $Gata3^{high}$ ) associated with low levels of Gata2 ( $Gata2^{low}$ ) (yellow arrow in Figure 27A-A" and Figure 29O). Surprisingly, we also identified a small fraction of V2b that did not co-express neither Gata2 nor Gata3 (arrowheads in Figure 27A-A" and Figure 29O). In the ventral part of the  $Gata2^+/Gata3^+$  population, part of the V2b subsets contained Onecut transcription factors (arrowheads in Figure 27G-G", Figure 29P-Q and data not shown). Although BhlhB5 was reported to not to be present in V2b at e10.5 (Skaggs et al., 2011), a subset of V2b located at the centre of the

---

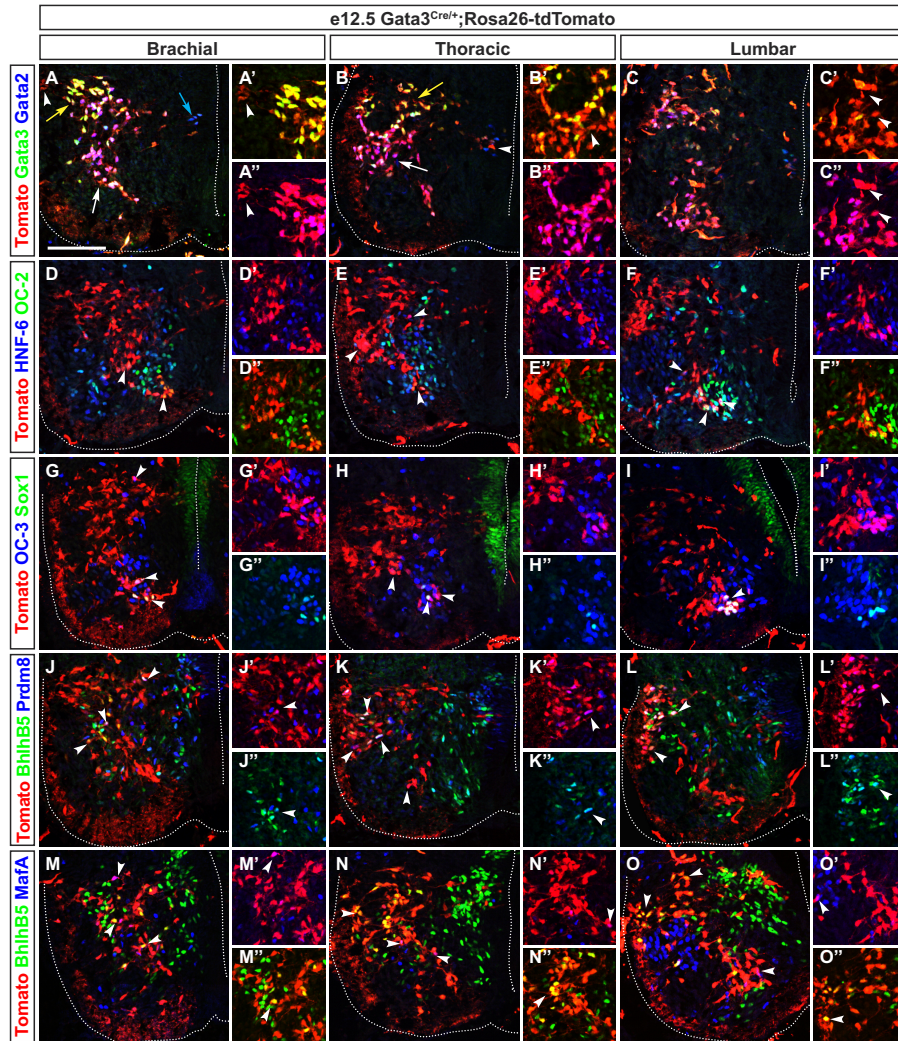
interneurons (arrows). MafA is detected in a few V2a cells that co-express Pou3F1 (white) as indicated by arrowhead. (H-H") At thoracic levels, Pou3F1 is detected in majority of V2a interneurons (arrows) whereas MafA remains restricted to a few cells. (I-I") At lumbar levels, Pou3F1 is present in a subset of V2a (yellow) located ventrally and close to motor neurons (arrow). MafA define a small subset of V2a that co-express Pou3F1 (white) and is located more ventrally, close to the floor plate (arrow). (J-J") At brachial levels, BhlhB5 define a subset of newly-born V2a interneurons (cyan) indicated by right arrowhead while Prdm8 is detected in another subset (yellow) located in the dorso-ventral flux of V2a interneurons as shown by left arrowhead. Prdm8 and BhlhB5 (white) are detected in a few V2a close to the central canal. (K-K") At thoracic levels, this population is expanded (arrowheads). (L-L") At lumbar levels, BhlhB5 is detected in subsets of V2a interneurons including newly-born V2a (cyan) and V2a in the dorso-ventral flux (arrowheads). Prdm8 is also detected in subsets within the dorso-ventral flux and in newly-born V2a (yellow) and is present in some BhlhB5<sup>+</sup> V2a (white). Scale bar = 100µm.

Gata2<sup>+</sup>/Gata3<sup>+</sup> subpopulation and close to the MNs contained Bhlhb5 (arrowheads in Figure 27J-J", Figure 29R). Prdm8 was present with Bhlhb5 in a part of these cells and was also detected in a distinct V2b subset (arrowheads in Figure 27J-J", Figure 29R). A last subset was characterized by the presence of MafA<sup>+</sup> (Figure 27M-M", Figure 29S). Finally, V2c INs characterized by the presence of Sox1 were located ventrally to the Gata2<sup>+</sup>/Gata3<sup>+</sup> V2b population and contained HNF-6, OC-2 and OC-3 (arrowheads in Figure 27G-G", Figure 29P-Q, and data not shown).

The distribution of V2a subsets was relatively stable at thoracic and at lumbar levels compared to brachial regions (Figure 26B-C", 4E-F", Figure 29K-N), with the exception of the Pou3F1 subset that expanded at thoracic levels and of the Prdm8<sup>+</sup> V2a neurons that were more numerous at thoracic and lumbar levels (Figure 26H-I", 4K-L", Figure 29M-N). Similarly, V2b and V2c subsets were very homogenous along the rostrocaudal axis of the spinal cord (Figure 27, Figure 29O-S and data not shown).

Taken together, these observations showed that V2 subpopulations can be divided into multiple subsets characterized by the combinatorial presence of HNF-6, OC-2, OC-3, MafA, cMaf, Pou3F1, Prdm8 or Bhlhb5. These subsets exhibited a rather homogenous distribution along the rostrocaudal axis of the spinal cord.





**Figure 27: Differential distribution of V2b interneuron subdivisions along the rostrocaudal axis of the spinal cord.** Immunofluorescence labeling on transverse section at brachial levels of the spinal cord of  $Gata3^{Cre/+}Rosa26^{floxstop-tdTomato}$  embryo at e12.5. (A-A'') V2b interneurons subdivide into a dorsal subset that contains high levels of Gata3 (yellow) indicated by yellow arrow, a ventral subset that contains Gata2 (magenta) indicated by white arrow and a subset of newly-born V2b wherein high Gata2 and low Gata3 levels are detected (blue arrow). (B-B'') At thoracic levels, low content in Gata2 (blue) is detected in a dorsal  $Gata3^+$  subset (yellow) indicated by yellow arrow while low content in Gata3 is detected in the  $Gata2^+$  subset (white) located ventrally (white arrow). (C-C'') At lumbar levels, Gata2 and Gata3 are present in a dorsal and in a ventral subset of V2b interneurons (white). (D-D'') At brachial levels, HNF-6 or OC-2 are present (magenta or yellow) or co-detected (white) in subsets of V2b interneurons (arrowheads). (E-E'') Similarly, at thoracic levels, HNF-6 and OC-2 are co-detected in a ventral subset of V2b interneurons (white) as indicated by bottom arrowhead. OC-2 is present in a few V2b (yellow) while HNF-6 defines a small lateral subset (magenta) indicated by left and top →

### Differential distribution of V3 interneuron subsets along the rostrocaudal axis of the spinal cord.

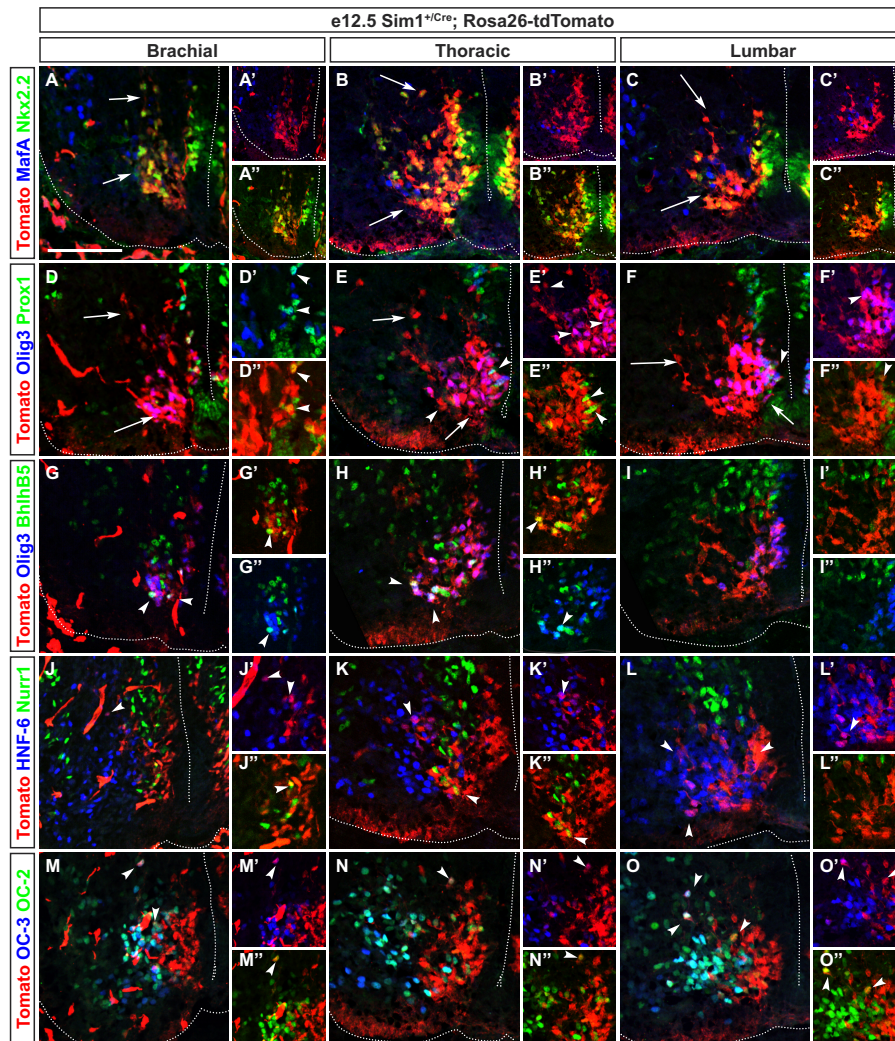
V3 INs characterized by Sim1 and Nkx2.2 expression arise from p3 progenitors defined by Nkx2.2 (Briscoe et al., 1999; Todd et al., 2012). These excitatory commissural neurons diversify into V3<sub>D</sub> and V3<sub>V</sub> according to their location and their respective electrophysiological characteristics (Zhang et al., 2008). However, markers for V3<sub>D</sub> or V3<sub>V</sub> cells have not been identified to date. To analyze the V3 INs, we relied on a Sim1<sup>Cre/+</sup>;Rosa26<sup>floxstop-tdTomato</sup> mouse line in which all V3 derivatives are permanently labeled by the expression of tdTomato. V3 INs initially gathered close to the floor plate and the central canal, and migrated according to a ventral to dorso-lateral stream and along a ventral to dorsal stream close to the midline (arrows in Figure 28A-C).

arrowheads. (F-F'') At lumbar levels, HNF-6 and OC-2 are also co-detected in a ventral subset of V2b interneurons (white) indicated by bottom arrowheads while OC-2 (yellow) or HNF-6 (magenta) alone defines small subsets of V2b (left arrowhead). (G-G'') At brachial levels, OC-3 is present in all V2c interneurons (bottom arrowheads), which contain Sox1 (white), and in a subset of V2b (magenta) located dorsally to V2c neurons. (H-I'') Similarly, at thoracic and lumbar levels, all V2c are OC-3<sup>+</sup> (white) and OC-3 is also present in another subset of V2b cells (arrowheads). (J-J'') At brachial levels, BhlhB5 defines a subset of V2b (yellow) close to motor neurons (left arrowhead). BhlhB5 and Prdm8 are co-detected in a few of these cells (white) as indicated by top arrowheads. (K-K'') At thoracic levels, BhlhB5 and Prdm8 are present in a subset of V2b (white) located dorsally to the motor neurons (left arrowheads). In addition, Prdm8 is detected in a small subset of V2b (magenta), which is located dorsally to BhlhB5<sup>+</sup>/Prdm8<sup>+</sup> V2b subset (left and bottom arrowheads). (L-L'') At lumbar levels, BhlhB5 and Prdm8 are present in a subset of V2b (white) located dorsally to the motor neurons (top arrowhead). Similarly to thoracic levels, Prdm8 is observed in subset of V2b (magenta) laterally and close to BhlhB5<sup>+</sup>/Prdm8<sup>+</sup> subset of V2b interneurons (bottom arrowhead). (M-M'') At brachial levels, MafA is detected in a small subset of V2b (magenta) indicated by top arrowhead whereas BhlhB5 defines another subset of V2b (yellow) indicated by arrowheads. (N-N'') At thoracic levels, the amount of V2b neurons that are MafA<sup>+</sup> (magenta) is decreased (right arrowhead) compared to brachial levels. V2b subset expressing BhlhB5 (yellow) is detected dorsally to the motor neurons (left arrowheads). (O-O'') At lumbar levels, fewer MafA<sup>+</sup> V2b cells (magenta) are observed than at brachial or thoracic levels (right arrowhead) while BhlhB5<sup>+</sup> V2b subset (yellow) is located dorsally to the motor neurons and close to the basal lamina (left arrowheads). Scale bar = 100µm.

First, we confirmed that Nkx2.2 was maintained in all V3 derivatives along the rostrocaudal axis of the spinal cord at e12.5 (Figure 28A-C) (Briscoe and Ericson, 1999). Then, at brachial levels, we observed that Olig3, a transcription factor which is present in p0, p2 and p3 progenitor domain (Takebayashi et al., 2002), was also detected in a large subset of V3 INs located ventrally and close to the central canal (arrow in Figure 28D-D" and Figure 29T), but not in more dorsal and/or migrating V3 INs (arrow in Figure 28D', 28E', 28F'). These cells could correspond to V3<sub>v</sub> INs. This population further subdivided into subsets characterized by the presence of Prox1, Bhlhb5 and Nurr1 (arrowheads in Figure 28D-D", 28G-G", 28J-J", Figure 29V). By contrast, members of the Onecut transcription factor family were detected in subsets of V3 INs located in a dorso-lateral stream, likely corresponding to V3<sub>d</sub> cells (arrowheads in Figure 28J-J", 28M-M", Figure 29V-W) and also in a few V3 neurons close to the floor plate that correspond to V3<sub>v</sub> cells.

At thoracic and lumbar levels, the location of V3 INs did not change significantly compared to brachial levels. Nonetheless, the amount of V3<sub>d</sub> INs gradually increased at the expense of the V3<sub>v</sub> INs that contained Olig3 (Figure 28D-I", Figure 29T-U). In addition, subsets containing Nurr1 or Bhlhb5 were expanded at thoracic levels (arrowheads in Figure 28K-K", Figure 28H-H", Figure 29U-V).

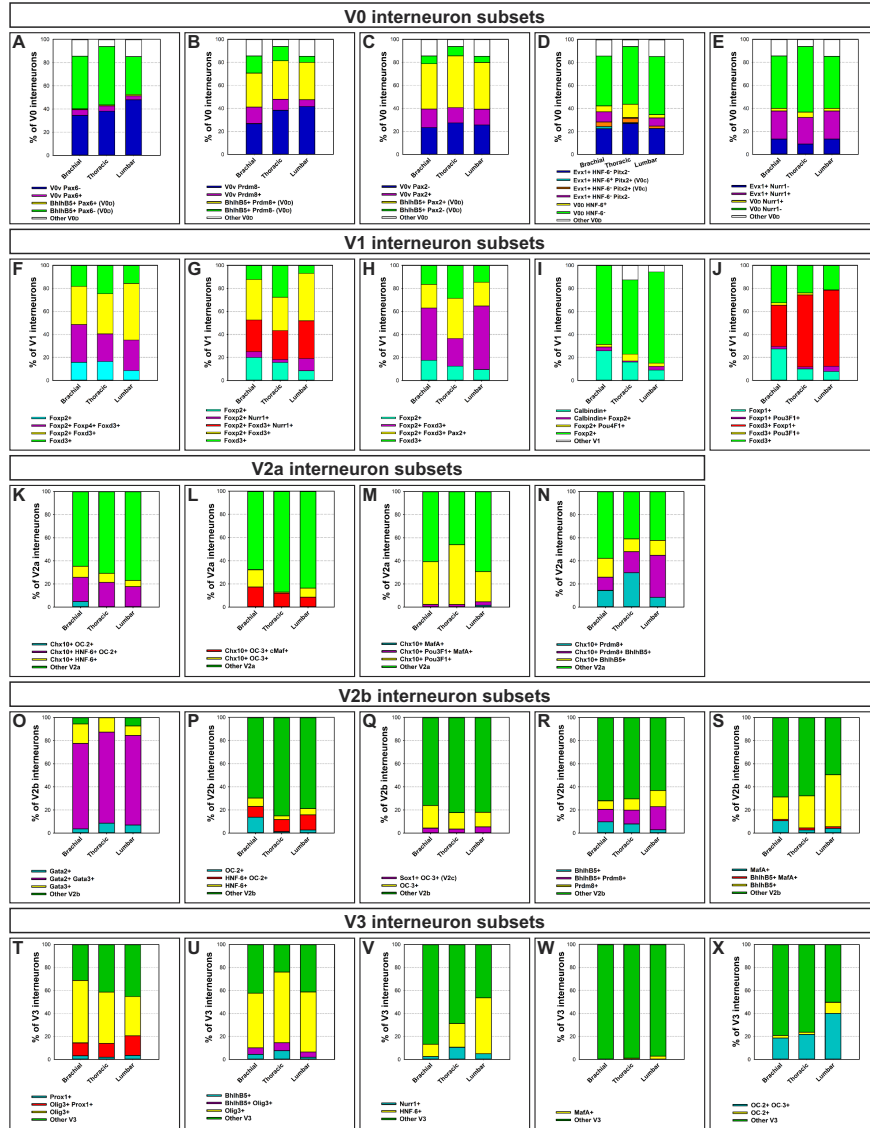
Together, these data indicate that the topographic distribution of V3 INs did not significantly differ at limbs or thoracic levels but that V3 subsets are differentially distributed along the rostrocaudal axis of the developing spinal cord. Most of these subsets are characterized by distinct combinations of transcription factors.



**Figure 28: V3 interneuron subsets are differentially distributed along the rostrocaudal axis of the spinal cord.** Immunofluorescence labeling on transverse section at brachial levels of the spinal cord of  $Sim1^{Cre/+}; Rosa26^{floxstop-tdTomato}$  embryo at e12.5. (A-A'') All the V3 interneurons contain Nkx2.2 (yellow) and subdivide in 2 groups (arrows), while MafA (magenta) is present in a few V3 cells. (B-B'') At thoracic levels, a majority of V3 neurons are labeled for Nkx2.2 (yellow) and very few of them contain MafA (white). (C-C'') Similarly, at lumbar levels, almost all V3 neurons (in red) are labeled for Nkx2.2 (yellow) and MafA define a very small subset of V3 neurons (white or magenta), located close to the floor plate. (D-D'') At brachial levels, a majority of V3 neurons located ventrally, possibly corresponding to  $V3_v$ , contain Olig3 (magenta) indicated by bottom arrow. In contrast, Prox1 is detected in very few V3 neurons (yellow or white) indicated by arrowheads. (E-E'') At thoracic levels, a large subset of V3 neurons located ventrally is defined by Olig3 (magenta) indicated by bottom arrow, a part of them containing Prox1 (white) indicated by right arrowhead. (F-F'') At lumbar levels, Olig3 is detected in more ventral V3 neurons (magenta) indicated by right arrow but not in V3 cells  
→

that migrate more dorsally indicated by left arrow, possibly corresponding to V3<sub>D</sub>. Prox1 is present in some V3<sub>V</sub> neurons (yellow or white) as shown by arrowhead. (G-G") At brachial levels, BhlhB5 is detected in some V3<sub>V</sub> Olig3<sup>+</sup> neurons (white) indicated by arrowheads. (H-H") At thoracic levels, the amount of V3<sub>V</sub> neurons that contain BhlhB5 (white) is increased (arrowheads). (I-I") At lumbar levels, BhlhB5 is detected neither in V3<sub>V</sub> nor V3<sub>D</sub>. (J-J") At brachial levels, HNF-6 is detected in migrating V3<sub>D</sub> neurons (magenta) indicated by arrowhead while Nurr1 is present in very few V3<sub>V</sub> neurons (yellow). (K-K") Similarly, at thoracic levels, HNF-6 is observed in migrating V3<sub>D</sub> neurons (magenta) indicated by left arrowhead whereas Nurr1 is present in a small subset of V3<sub>V</sub> neurons (yellow) indicated by right arrowhead. (L-L") At lumbar levels, HNF-6 is detected in migrating V3<sub>D</sub> neurons (magenta) indicated by left arrowheads while Nurr1 is absent from V3 neurons (see right arrow). (M-M") At brachial levels, OC-2 and OC-3 are co-detected in all V3<sub>D</sub> neurons (white) indicated by top arrowhead while a few V3<sub>V</sub> are labeled for OC-2 only (yellow) as indicated by bottom arrowhead. (N-N") Similarly, at thoracic levels, OC-2 and OC-3 are present in all V3<sub>D</sub> neurons (white) indicated by arrowhead while OC-2 alone is detected in a small subset of V3<sub>V</sub> (yellow). (O-O") At lumbar levels, a large subset of V3<sub>D</sub> contain OC-2 and OC-3 (white) indicated by top arrowheads, but OC-2 is also present in subsets of V3<sub>D</sub> and V3<sub>V</sub> neurons (yellow) as shown by right arrowhead. Scale bar = 100µm.





**Figure 29: Quantification of ventral interneuron subsets along the anteroposterior axis of the spinal cord.** (A-X) Histograms of quantification of V0, V1, V2a, V2b and V3 interneuron subsets at brachial, thoracic and lumbar levels of the spinal cord of mouse embryos at e12.5 show distinct distribution and proportion of different subsets within each interneuron cardinal classes. Histograms are normalized according to the total amount of cells in each ventral IN population, which corresponds to 100% (n=3).

## Results

| Interneuron Classes | Subpopulations   |                                               | Subset markers                                               |
|---------------------|------------------|-----------------------------------------------|--------------------------------------------------------------|
| <b>V0</b>           | V0 <sub>D</sub>  | BhlhB5                                        | Pax2 > Prdm8 > Nurr1                                         |
|                     | V0 <sub>V</sub>  | Evx1                                          | Pax2>OC3>Nurr1>Prdm8>Pax6<br>HNF-6/OC-2 > MafA               |
|                     | V0 <sub>CG</sub> | Evx1, Pitx2                                   | HNF-6                                                        |
| <b>V1</b>           | Renshaw cells    | Calbindin, OC <sup>a</sup> , Foxd3, En1, MafB |                                                              |
|                     | Ia INs           | Foxp2, Foxd3, En1                             | Foxp4 > Pax2 > Arx > Nurr1<br>BhlhB5,Prdm8> Prdm8 > Pou4F1   |
|                     | Other V1         | Foxd3, En1                                    | Foxp1 > Pou3F1<br>Nurr1 > BhlhB5 > Prdm8                     |
| <b>V2</b>           | V2a              | Chx10                                         | BhlhB5 > Pou3F1<br>OC <sup>a</sup> > Prdm8 > MafA > cMaf     |
|                     | V2b              | Gata3, Gata2                                  | BhlhB5 > BhlhB5/Prdm8 > Prdm8<br>OC <sup>a</sup> > MafA/MafB |
|                     | V2c              | Sox1, OC <sup>a</sup>                         |                                                              |
| <b>V3</b>           | V3 <sub>D</sub>  | OC <sup>a</sup>                               |                                                              |
|                     | V3 <sub>V</sub>  | Olig3                                         | Prox1 > BhlhB5<br>Nurr1                                      |

**Table 5. Summary of ventral interneuron subsets at e12.5.** Summary of the subdivisions of ventral interneuron classes, according to the expression of the studied markers (from more numerous to less numerous cells). <sup>a</sup> The three Onecut factors

### **3.1.4. Discussion**

Despite the identification of four cardinal classes of ventral spinal INs and the discovery of some of the molecular and genetic mechanisms that control their specification, little is known about their respective spatial distribution along the rostrocaudal axis of the spinal cord and their diversification into multiple subsets. Here, we provided evidence that each class of ventral INs displays distinct columnar organization that varies at brachial, thoracic and lumbar levels of the developing spinal cord. In addition, we showed that the V0, V1, V2 and V3 cardinal classes of INs can be subdivided into several subpopulations or subsets, based on the combined expression of multiple transcription factors (Table 5). Hence, these findings provide a basis for a better characterization of the diversification of embryonic ventral INs, and for investigating the physiology and function of ventral INs subsets in the spinal motor circuits.

#### **Topographic and columnar organization of ventral spinal INs.**

The differential distribution of ventral IN populations and subsets along the rostrocaudal axis of the spinal cord likely correlates with the various functions of local spinal neuronal circuits that regulate body movements or locomotive behaviors. Indeed, thoracic circuits ensure control of body wall musculature, whereas brachial and lumbar neuronal populations additionally provide regulation of limb movements. Consistently, the composition in ventral IN subsets varies along the rostrocaudal axis of the spinal cord, suggesting that the local pre-motor circuits at brachial, thoracic or lumbar levels are shaped to support these distinct activities. The exact position and contribution of each IN subset within these pre-motor circuits, including its direct or indirect relationship to MNs, remains to be determined. Surprisingly, the differences in the composition of vIN subsets



between brachial and lumbar levels were as important as between these limb levels and thoracic levels. This suggests that the organization of the locomotor circuits and the strategies to control limb movements are quite different between the brachial and the lumbar region. This observation may be correlated with the distinct composition in motor pools between these two regions (Dasen et al., 2005; De Marco Garcia and Jessell, 2008; Lin et al., 1998).

Previous studies have established that spinal MNs also exhibit a differential organization along the rostrocaudal axis of the spinal cord (Dasen et al., 2003; Tsuchida et al., 1994). Studies of the developmental mechanisms that control the topographic and columnar organization of MNs reveal roles for extrinsic factors such as RA, FGFs and Wnts in regulating Hox gene expression (Dasen et al., 2008; Dasen et al., 2003; Liu et al., 2001; Nordstrom et al., 2006; Rousso et al., 2008). As per motor neurons, the topographic organization of ventral INs may be regulated by RA, FGFs or Wnts, either directly or through combined activities of Hox genes. Indeed, RA from the paraxial mesoderm appears to be sufficient to specify and promote the generation of V0, V1 and V2 INs (England et al., 2011; Pierani et al., 1999; Renoncourt et al., 1998; Wilson et al., 2004). In addition, LMC MNs constitute another source of RA that could influence the differentiation and distribution of V0, V1 and V2 INs at brachial and lumbar levels (Rousso et al., 2008; Sockanathan and Jessell, 1998; Sockanathan et al., 2003; Vermot et al., 2005). However, the potential role of RA in the distribution of V0, V1 and V2 INs along the rostrocaudal axis has not been investigated yet. Previous studies have also shown that the Hox genes involved in the rostrocaudal patterning of MNs and in the specification of motor neuron pools, namely Hoxc6 and Hoxc8 at brachial levels, Hoxc9 and Hoxd9 at thoracic levels and Hoxa10, Hoxc10 and Hoxd10 at lumbar levels, are also expressed in ventral INs (Jung et al., 2010; Vermot et al., 2005; Wu et al., 2008). Moreover, the Hox co-factor Foxp1 is expressed in V1 INs,

while Pbx1 and Prep/Meis are more broadly expressed in ventral INs (Cooper et al., 2003; Karlsson et al., 2010; Moens and Selleri, 2006; Roberts et al., 1995). This raises the possibility that these co-factors partner with Hox proteins to differentially regulate ventral IN diversification. The Hox genes may therefore coordinately regulate rostrocaudal distribution and columnar organization of ventral INs and of MNs in the developing spinal cord.

### ***Control of the diversification of ventral INs***

With the exception of studies that show Scl and Dll4/Notch signaling control the generation of V2a and V2b cells from V2 progenitors (Del Barrio et al., 2007; Joshi et al., 2009; Peng et al., 2007), we still know very little about the molecular mechanisms and factors that regulate ventral INs diversification. Several studies have unveiled that extrinsic factors including Sonic hedgehog, BMPs, FGFs, Wnts or RA are involved in the specification and differentiation programs of several neuronal populations within the spinal cord (Dessaud et al., 2008; Lee et al., 2009; Novitsch et al., 2003). Our study describes a number of developmentally regulated transcription factors that subdivide three cardinal classes of ventral INs into several subsets. Interestingly, some of these factors might be downstream of FGF or RA signaling (Dasen et al., 2005; De Marco Garcia and Jessell, 2008; Francius and Clotman, 2010; Wu et al., 2008). Indeed, the expression of Nurr1, which labels some subsets in V0<sub>v</sub>, V1 non-RC neurons or V3<sub>v</sub> INs, is regulated by FGFs in the mesencephalon (Baron et al., 2012; Grothe et al., 2004; Lammi and Aarnisalo, 2008) and by RA signaling in the ventral spinal cord (Skaggs et al., 2011). Similarly, BhlhB5, which defines several subsets within V0, V1, V2b and V3 INs, is modulated by RA in the same tissue (Skaggs et al., 2011). Furthermore, Pou3F1, which defines subsets within V1 and V2a INs, is regulated by Hoxc8 in the motor pool that

innervates the flexor carpi ulnaris muscle (Dasen et al., 2005). The Onecut factors, which are detected within  $Nkx6.1^+$ ,  $Pea3^+$  or  $Pou3F1^+$  motor pools (Francius and Clotman, 2010) and may therefore be downstream of Hox genes, are also expressed within several subsets of V0, V2 and V3 INs (at brachial or lumbar levels). Thus, the mechanisms that ensure proper rostrocaudal patterning of the motor neurons, including FGF or RA pathways and Hox genes, may additionally contribute to the diversification of ventral spinal IN subsets, as previously suggested for Hox4-8 genes (Jung et al., 2010).

In addition, the transcription factors identified in the present study as markers of ventral IN subsets may participate in the control of their diversification. Onecut factors have been recently shown to participate in the development of RCs (Stam et al., 2012). Similarly, BhlhB5 plays a crucial role in the differentiation of early-born V2 INs (Skaggs et al., 2011). Surprisingly, we identified BhlhB5 as a marker of most of the  $V0_D$  INs at e12.5, although previous studies have reported that BhlhB5 is not expressed in V0 cells at e10.5 (Brunelli et al., 2003; Liu et al., 2007; Skaggs et al., 2011). This suggests that Bhlhb5 expression may be activated in a majority of  $V0_D$  INs later than e10.5. Alternatively, as recently demonstrated for V1 INs development that involves successive waves of neurogenesis (Benito-Gonzalez and Alvarez, 2012; Stam et al., 2012), V0 progenitors may give rise to  $V0_V$  (devoid of Bhlhb5) prior to  $V0_D$  cells (that express BhlhB5). Given the importance of Bhlhb5 for the development of other spinal populations (Ross et al., 2010; Ross et al., 2012; Skaggs et al., 2011), it also indicates that Bhlhb5 could participate in further diversification of  $V0_D$  cells. In addition, we identified a large  $BhlhB5^+$  subset within the V2b subpopulation whereas it has been reported that BhlhB5 is selectively expressed at e10.5 in V2a but not in V2b neurons (Skaggs et al., 2011). Further analyses of BhlhB5 function using loss or gain-of-function experiments are needed to clarify the role of BhlhB5 during  $V0_D$  and V2 INs development. Finally,

Nurr1 is expressed in glutamatergic cortical neurons (Andjelic et al., 2009; Arimatsu et al., 2003) and in glutamatergic spinal INs (unpublished data), which suggests that Nurr1 may participate in the differentiation of glutamatergic (neurons including V0<sub>v</sub> and V3) INs (Li et al., 2011).

Furthermore, most of the identified markers of IN subsets are expressed in a combinatorial manner and may interact to promote specific neuronal fate. For example, BhlhB5 and Prdm8, which shares numerous target genes, cooperate during differentiation of dl6, V1 and V2a INs (Liu et al., 2007; Skaggs et al., 2011; Stam et al., 2012) and of dorsal INs (Ross et al., 2010; Ross et al., 2012). We have shown that Prdm8 is co-expressed with BhlhB5 in some subsets of V0<sub>d</sub>, V1 and V2 INs. However, we also identify Prdm8<sup>+</sup> subsets that do not express BhlhB5 and vice versa. Among the identified common target genes of BhlhB5 and Prdm8, the type II cadherin-11 (Cdh-11) (Ross et al., 2012) has been reported to regulate different aspects of spinal motor neuron development including differentiation, columnar organization (Marthiens et al., 2002), axonal growth, migration and fasciculation (Boscher and Mege, 2008; Marthiens et al., 2005). At e12.5, Cdh-11 is transiently expressed in ventral INs (Marthiens et al., 2002). Hence, upon BhlhB5 and Prdm8 regulation, Cdh-11 may participate in the diversification of ventral INs subsets.

Finally, numerous studies have revealed that Foxp factors control different steps of neuronal development. For example, Foxp2 regulates neuronal network formation (Rousso et al., 2012; Vernes et al., 2011), radial and/or tangential migration of cortical interneurons (Clovis et al., 2012), neurite outgrowth (Vernes et al., 2011) as well as differentiation of several neuronal populations within the ventral spinal cord including MNs, V0<sub>v</sub> and V2 INs (Rousso et al., 2012). Foxp2 is also expressed from e11.5 within large subpopulations of non-RC V1 neurons including Ia INs (Morikawa et al., 2009a) and may therefore control different

aspects of their development. Likewise, *Foxp4*, which is present in *Foxp2*<sup>+</sup> V1 cells, may participate in the regulation of differentiation, migration and axonal growth by cooperating with *Foxp2* as recently described (Li et al., 2004; Takahashi et al., 2008; Tam et al., 2011). *Foxp1* is also detected in subsets of V1 INs (Morikawa et al., 2009a; Tamura et al., 2003), suggesting that different combinations of *Foxp* factors may regulate distinct aspects of V1 development including differentiation and migration as recently suggested (Hisaoka et al., 2010; Morikawa et al., 2009a; Morikawa et al., 2009b).

### **3.1.5. Conclusion and perspectives.**

In this study, we showed that ventral spinal IN populations are differentially distributed along the rostrocaudal axis of the spinal cord, and can be subdivided into multiple subsets according to a combinatorial code of transcriptional regulators. This characterization constitutes a resource that will facilitate further studies aimed at understanding the mechanisms that drive ventral spinal IN diversity during embryonic development. It will also provide a foundation for establishing the relationship between these embryonic subsets and the multiple IN populations that populate the adult spinal cord.

Recent advances in the engineering of powerful genetically-modified biological tools, such as genetic lineage tracing mouse lines, modified trans-synaptic tracing viruses, conditional neuronal silencing and optogenetic systems (Grossmann et al., 2010a; Osakada et al., 2011; Wall et al., 2010) have greatly contributed to the improvement of our knowledge of neural circuitry and their physiological functions. Hence, our data will contribute to design strategies to genetically manipulate these neuronal subsets. Such experiments should allow to further decipher the organization of the spinal neural circuits that regulate locomotive

---

## **Results**

behaviors and to elucidate in more details the different functions of these cells in the neonate and adult spinal cord.

### 3.1.6. Materials and Methods

#### Animals

All experiments were strictly performed respecting the European Community Council directive of 24 November 1986 (86-609/ECC) and the decree of 20 October 1987 (87-848/EEC). CD1 mice were raised in our animal facility and were treated according to the principles of laboratory animal care of the Animal Welfare Committee of the Université catholique de Louvain (Permit Number: UCL/MD/2009/008). The day of vaginal plug was considered as embryonic day (e)0.5 and embryos were collected at e12.5. Cohorts of a minimum of 3 embryos per experiment were used.

The *Dbx1*<sup>LacZ/+</sup>, *En1*<sup>Cre/+</sup>;ZnG, *Sim1*<sup>Cre/+</sup>;Rosa26<sup>floxstop-tdTomato</sup> mutant mice were previously described (Pierani et al., 2001; Sapir et al., 2004; Zhang et al., 2008). The specificity controls of b-galactosidase, Cre recombinase or GFP expression for each mouse line were provided in the cited references. *Gata3*<sup>Cre/+</sup>;Rosa26<sup>floxstop-tdTomato</sup> mice were generated by breeding *Gata3*<sup>Cre/+</sup> mice (Zhang et al., submitted) with Rosa26<sup>floxstop-tdTomato</sup> mice. Characterization of *Gata3*<sup>Cre/+</sup>;Rosa26<sup>floxstop-tdTomato</sup> mouse line was provided in the cited reference. In addition, our immunofluorescent analyses in the developing spinal cord indicated that Cre recombinase and tdTomato expression was restricted to the V2b and V2c interneurons (data not shown).

Mouse embryos were collected at e12.5 and decapitated before fixation. Embryos were fixed in PBS/4% PFA at 4°C for 25 minutes. They were washed thrice in cold PBS before incubation in PBS/30% sucrose overnight at 4°C. They were embedded in PBS/7.5% gelatin/15% sucrose before being frozen at -55°C. Embryos were cut at 14µm in a Leica CM3050 cryostat and cryosections were stored at -20°C. For whole mount experiments, the whole spinal cord was removed, fixed in cold PBS/4% PFA for 25 minutes and were processed immediately.

### **Immunofluorescent labelings**

Cryosections or whole spinal cord were saturated with PBS/0.1% Triton/10% horse serum for 30 minutes and incubated with the primary antibodies diluted in the same solution at 4°C overnight or for 4 days, respectively. After 3 washes in PBS/0.1% Triton, the secondary antibodies, diluted in PBS/0.1% Triton/10% horse serum, were added for 30 minutes at room temperature. Slides or whole spinal cord were washed thrice in PBS/0.1% Triton before a final wash in PBS, and were mounted with Fluorescent mounting medium (DAKO).

Details of the sources of the primary or secondary antibodies, and their characterization by us or by others are indicated in Table 3 and Table 4, respectively. All secondary antibodies gave signals in the spinal cord only in the presence of corresponding primary antibodies, but not when applied on spinal cord sections alone.

### **Imaging and quantitative analyses**

Immunofluorescence images of cryosections were acquired on a Zeiss Axio Cell Observer Z1 confocal microscope with the Zeiss AxioVision Rel. 4.8 software and processed with Adobe Photoshop CS5 software.

Quantifications were performed on red or green or blue layer of acquired confocal images and double or triple labeling cells were processed by subtractive method (Francius and Clotman, 2010). For each e12.5 embryo (n=3), labeled cells were quantified using the count analysis tool of Adobe Photoshop CS5 software. Neurons that were positive for molecular markers described above were counted unilaterally on three sections at three different levels of the brachial, thoracic and lumbar regions. Depending on the rostrocaudal level, the amount of cells that was counted for each IN population was: 91-219 cells for V0, 169-257 cells for V1, 50-107 cells for V2a, 59-103 cells for V2b and 41-76 cells for V3. Raw data were



exported from Adobe Photoshop CS5 software to SigmaPlot v12 software and processed in order to generate histogram figures. Analyses of the Variance performed for each experiment by One Way ANOVA test indicated a low and non-significant variability from mouse to mouse ( $0.053 < p < 0.065$ ). Each histogram generated in this study was normalized to the total amount of cells in each ventral interneuron population that was set at 100.

Z-stack images of whole mount labelings in open-book spinal cord preparations were obtained using LSM510 and Zeiss Axio Cell Observer Z1 confocal microscope. Three-dimensional (3D) image reconstructions were made and processed using Zeiss ZEN 2011 black edition (release version 7.0.0.285) software. Optical sections in Z-series collections were collected at  $0.540\mu\text{m}$  intervals (objective: LD LCI Plan-Apochromat 25x0.8 Imm Korr DIC M27, camera: AxioCamMR3). Brightness and contrast were modified using Zeiss ZEN 2011 software, then movies were generated with Zeiss ZEN 2011 software.

### 3.1.7. Acknowledgments

We thank people of the NEDI lab for technical help, reagents and discussions, and Emma Martinelly for editing the manuscript. We are grateful to Dr. Sarah E. Ross, Dr. Yuichi Ono, Dr. Stavros Malas, Dr. Kamal Sharma, Dr. Sam Pfaff, Dr. Thomas Müller and Dr. Carmen Birchmeier for kindly providing with the rabbit and rat anti BhlhB5, rabbit and guinea pig anti Prdm8, rat anti Nurr1, rabbit and guinea pig anti Sox1, rabbit anti Chx10 and guinea pig anti Gata2/3, guinea pig anti Chx10, rabbit and guinea pig anti Lbx1, anti-Olig3, guinea pig anti MafA, rabbit anti MafB, anti cMaf and the guinea pig anti-Foxd3 antibodies, respectively. The monoclonal antibody anti Evx1 (99.1-3A2) and anti Nkx2.2 (74.5A5) developed by Thomas M. Jessell the monoclonal antibody anti Isl1/2 (39.4D5) developed by Thomas M. Jessell and Susan Brenner-Morton and anti Pax6 (PAX6) developed by Atsushi Kawakami were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biology, Iowa City, IA 52242.

This work was supported by a grant from the "Fonds spéciaux de recherche" (FSR) of the UCL, by a "Crédit au chercheur" #1.5.085.10.F and a grant from the Fund for Scientific Medical Research #3.4.538.10.F of the F.R.S.-FNRS, by the "Association Belge contre les Maladies neuro-Musculaires asbl" (ABMM) and by the "Actions de Recherche Concertées (ARC)" #10/15-026 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'" to F.C., M.B. was the recipient of a fellowship from NeRF (Neuropôle de recherche francilien). A.P. is a CNRS (Centre National de la Recherche Scientifique) Investigator, C.F. is supported by the FSR (UCL). F.C. is a Research Associate of the F.R.S.-FNRS.

## Results

| Primary antibody used        | Dilution | Supplier            | Reference                   |
|------------------------------|----------|---------------------|-----------------------------|
| Rabbit anti-Arx              | 1:1000   | Dr. Jamel Chelly    | (Poirier et al., 2004)      |
| Chicken anti-b-galactosidase | 1:2000   | Abcam               | ab9361                      |
| Goat anti-BhlhB5             | 1:1000   | Santa Cruz          | sc-6045                     |
| Rabbit anti-BhlhB5           | 1:2000   | Dr. Sarah E. Ross   | (Ross et al., 2012)         |
| Rat anti-BhlhB5              | 1:2000   | Dr. Sarah E. Ross   | (Ross et al., 2012)         |
| Mouse anti-Brn3a             | 1:500    | Santa Cruz          | sc-8429                     |
| Guinea pig anti-Brn3a        | 1:5000   | Dr. Jane Johnson    | (Kim et al., 2008)          |
| Rabbit anti-Chx10            | 1:5000   | Dr. Kamal Sharma    | (Peng et al., 2007)         |
| Sheep anti-Chx10             | 1:500    | Exalpha Biologicals | X1179P                      |
| Guinea pig anti-Chx10        | 1:3000   | Dr. Sam Pfaff       | (Peng et al., 2007)         |
| Mouse anti-Calbindin         | 1:10000  | Swant               | CB300                       |
| Rabbit anti-Calbindin        | 1:10000  | Swant               | CB38                        |
| Mouse anti-Evx1              | 1:2000   | DSHB                | 99.1-3A2                    |
| Rabbit anti-Evx1             | 1:300    | Dr. Martyn Goulding | (Moran-Rivard et al., 2001) |
| Rabbit anti-Foxd3            | 1:5000   | Dr. Thomas Müller   | (Muller et al., 2005)       |
| Guinea pig anti-Foxd3        | 1:5000   | Dr. Thomas Müller   | (Muller et al., 2005)       |
| Goat anti-Foxp1              | 1:1000   | R&D Systems         | AF4534                      |
| Goat anti-Foxp2              | 1:2000   | Abcam               | ab1307                      |
| Rabbit anti-Foxp4            | 1:400    | Genway Biotech      | 18-003-43528                |
| Guinea pig anti-Gata2        | 1:3000   | Dr. Kamal Sharma    | (Peng et al., 2007)         |
| Mouse anti-Gata3             | 1:400    | Santa Cruz          | sc-268                      |
| Rat anti-Gata3               | 1:20     | Dr. Frank Grosveld  | (Panayi et al., 2010)       |
| Chicken anti-GFP             | 1:2000   | Abcam               | ab290                       |
| Rabbit anti-GFP              | 1:2000   | Aves Labs           | GFP-1020                    |
| Guinea pig anti-HNF-6        | 1:6000   | Our laboratory      | (Espana and Clotman, 2012b) |
| Rabbit anti-HNF-6            | 1:50     | Santa Cruz          | sc-13050                    |
| Rabbit anti-Lbx1             | 1:5000   | Dr. Thomas Müller   | (Muller et al., 2002)       |
| Guinea pig anti-Lbx1         | 1:5000   | Dr. Thomas Müller   | (Muller et al., 2002)       |

## Results

|                           |         |                    |                         |
|---------------------------|---------|--------------------|-------------------------|
| Guinea pig anti-MafA      | 1:10000 | Dr. Thomas Müller  | (Gierl et al., 2006)    |
| Rabbit anti-MafA          | 1:500   | Novus Biological   | NB400-137               |
| Rabbit anti-MafB          | 1:5000  | Dr. Hagen Wende    | (Gierl et al., 2006)    |
| Rabbit anti-cMaf          | 1:5000  | Dr. Hagen Wende    | (Wende et al., 2012)    |
| Mouse anti-Nkx2.2         | 1:20    | DSHB               | 74.5A5                  |
| Rat anti-Nurr1            | 1:2000  | Dr. Yuichi Ono     | (Ono et al., 2007)      |
| Rat anti-OC-2             | 1:400   | Our laboratory     | (Clotman et al., 2005)  |
| Guinea pig anti-OC-3      | 1:6000  | Our laboratory     | (Pierreux et al., 2004) |
| Rabbit anti-Olig3         | 1:5000  | Dr. Thomas Müller  | (Muller et al., 2005)   |
| Guinea pig anti-Olig3     | 1:5000  | Dr. Thomas Müller  | (Muller et al., 2005)   |
| Rabbit anti-Pax2          | 1:100   | Covance            | PRB-276P                |
| Goat anti-Pax6            | 1:500   | Santa Cruz         | sc-7750                 |
| Rabbit anti-Pax6          | 1:2000  | Covance            | PRB-278P                |
| Mouse anti-Pax6           | 1:1000  | DSHB               | PAX6                    |
| Rabbit anti-Pitx2         | 1:5000  | Capra Science      | PA 1020-100             |
| Rabbit anti-Prdm8         | 1:200   | Pr. Yoichi Shinkai | (Komai et al., 2009)    |
| Mouse anti-Prdm8          | 1:200   | Pr. Yoichi Shinkai | (Komai et al., 2009)    |
| Guinea pig anti-Prdm8     | 1:2000  | Dr. Sarah E. Ross  | (Ross et al., 2012)     |
| Rabbit anti-Prdm8         | 1:2000  | Dr. Sarah E. Ross  | (Ross et al., 2012)     |
| Rabbit anti-Prox1         | 1:1000  | Covance            | PRB-238C                |
| Goat anti-Prox1           | 1:500   | R&D Systems        | AF2727                  |
| Rabbit anti-RFP           | 1:2000  | Rockland Inc.      | 600-401-379             |
| Goat anti-Sox1            | 1:500   | Santa Cruz         | sc-17318                |
| Rabbit anti-Sox1          | 1:400   | Dr. Stavros Malas  | (Panayi et al., 2010)   |
| Guinea pig anti-Sox1      | 1:400   | Dr. Stavros Malas  | (Panayi et al., 2010)   |
| Rabbit anti-SCIP (Pou3f1) | 1:500   | Dr. Dies Meijer    | (Jaegle et al., 2003)   |

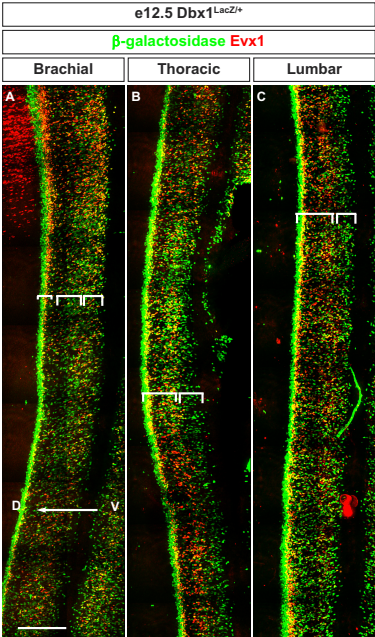
**Table 3: List of the primary antibodies used in this study**

## Results

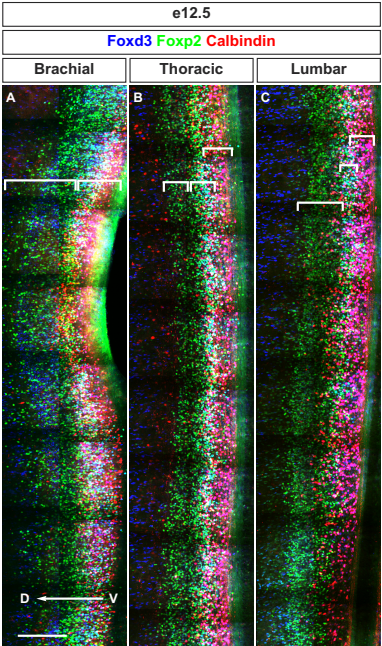
| Secondary antibody used                          | Dilution | Supplier               | Reference   |
|--------------------------------------------------|----------|------------------------|-------------|
| Donkey anti-mouse/AlexaFluor488                  | 1:2000   | Life Science           | A21202      |
| Donkey anti-mouse/AlexaFluor594                  | 1:2000   | Life Science           | A21203      |
| Donkey anti-mouse/AlexaFluor647                  | 1:2000   | Life Science           | A31571      |
| Donkey anti-rabbit/AlexaFluor647                 | 1:2000   | Life Science           | A31573      |
| Donkey anti-rat/AlexaFluor594                    | 1:2000   | Life Science           | A21209      |
| Donkey anti-goat/AlexaFluor488                   | 1:2000   | Life Science           | A11055      |
| Donkey anti-sheep/AlexaFluor594                  | 1:2000   | Life Science           | A11016      |
| Goat anti-guinea pig/AlexaFluor594               | 1:2000   | Life Science           | A11076      |
| Goat anti-mouse IgG <sub>1</sub> /AlexaFluor594  | 1:2000   | Life Science           | A21125      |
| Goat anti-mouse IgG <sub>2a</sub> /AlexaFluor488 | 1:2000   | Life Science           | A21131      |
| Donkey anti-chicken/Dylight488                   | 1:1000   | Jackson ImmunoResearch | 703-485-155 |
| Donkey anti-chicken/Dylight594                   | 1:1000   | Jackson ImmunoResearch | 703-515-155 |
| Donkey anti-mouse/AlexaFluor647                  | 1:1000   | Jackson ImmunoResearch | 715-605-151 |
| Donkey anti-rat/ AlexaFluor647                   | 1:1000   | Jackson ImmunoResearch | 712-605-153 |
| Donkey anti-sheep/AlexaFluor594                  | 1:1000   | Jackson ImmunoResearch | 713-585-147 |
| Donkey anti-sheep/AlexaFluor647                  | 1:1000   | Jackson ImmunoResearch | 713-605-147 |
| Donkey anti-rabbit/AlexaFluor488                 | 1:1000   | Jackson ImmunoResearch | 711-545-152 |
| Donkey anti-rabbit/AlexaFluor594                 | 1:1000   | Jackson ImmunoResearch | 711-585-152 |
| Donkey anti-guinea pig/AlexaFluor488             | 1:1000   | Jackson ImmunoResearch | 706-545-148 |
| Donkey anti-guinea pig/AlexaFluor647             | 1:1000   | Jackson ImmunoResearch | 706-605-148 |

**Table 4: List of the secondary antibodies used in this study**

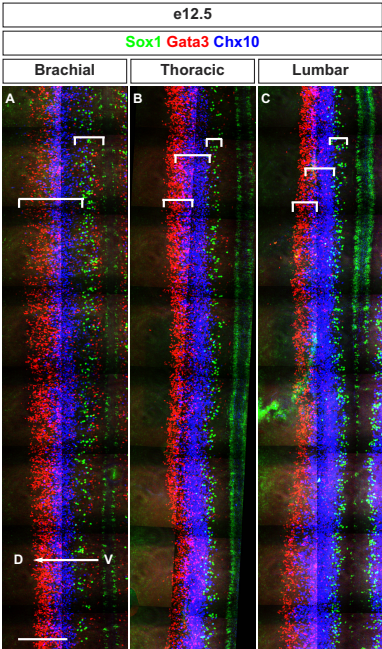
3.1.8. Supplementary figures



**Figure S1. Topographic distribution of V0 interneurons at e12.5.**  
(A-C) Whole-mount immunofluorescence analysis on spinal cord of Dbx1<sup>LacZ/+</sup> embryo at e12.5 shows differential distribution of V0 interneurons including V0<sub>v</sub> (Evx1<sup>+</sup>/b-galactosidase<sup>+</sup>) in red or yellow and V0<sub>d</sub> in green at brachial (A), thoracic (B) or lumbar (C) levels. The white arrow indicates ventral (V) to dorsal (D). Scale bar = 500μm.

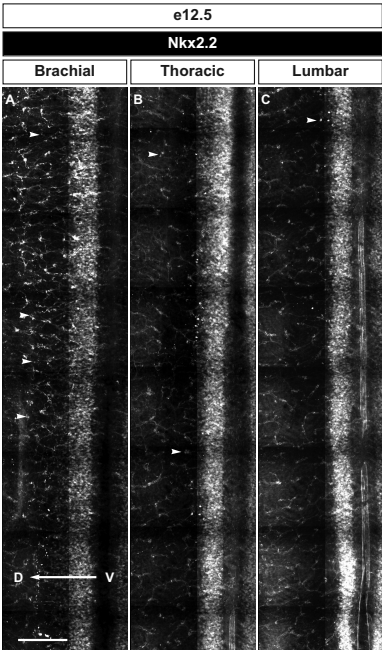


**Figure S2. Topographic distribution of V1 interneurons at e12.5.**  
(A-C) Whole-mount immunofluorescence analysis on spinal cord of embryo at e12.5 that shows differential distribution pattern of V1 interneurons including Renshaw cells (red) and V1 Foxp2<sup>+</sup> interneurons (green) at brachial (A), thoracic (B) or lumbar (C) levels. White arrowhead indicates ventral (V) to dorsal (D). Scale bar = 500μm.



**Figure S3. Topographic distribution of V2 interneurons at e12.5.**

(A-C) Whole-mount immunofluorescence analysis on spinal cord of embryo at e12.5 that shows differential distribution pattern of V2 interneurons including V2a (blue), V2b (red) and V2c (green) at brachial (A), thoracic (B) or lumbar (C) levels. White arrowhead indicates ventral (V) to dorsal (D). Scale bar = 500µm.



**Figure S4. Topographic distribution of V3 interneurons at e12.5.**

(A-C) Whole-mount immunofluorescence analysis on spinal cord of embryo at e12.5 that shows homogenous distribution pattern of V3 interneurons (white) at brachial (A), thoracic (B) or lumbar (C) levels. White arrowhead indicates ventral (V) to dorsal (D). Scale bar = 500µm.

Results

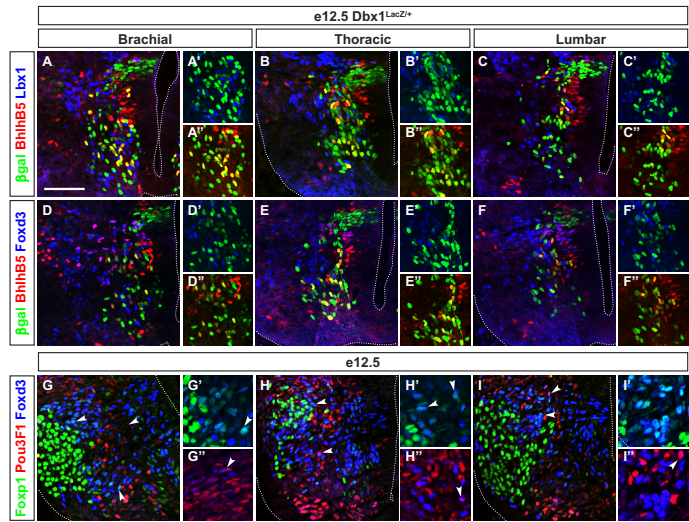


Figure S5. **V0<sub>D</sub> interneurons contain BhlhB5 at e12.5.** (A-C") Immunofluorescence labeling on transverse section at brachial (A-A"), thoracic (B-B") and lumbar (C-C") levels of the spinal cord of Dbx1<sup>LacZ/+</sup> embryo at e12.5 shows that V0<sub>D</sub> interneurons contain BhlhB5 (yellow) but not Lbx1<sup>+</sup> (blue), a marker of dorsal interneurons. (D-F") V0<sub>D</sub> interneurons that contain BhlhB5 (yellow) are not labeled for Foxd3<sup>+</sup> (blue), a marker of V1 interneurons. Hence, in Dbx1<sup>LacZ/+</sup> embryo, b-galactosidase is detected only in V0 interneurons and absent from V1 or dI6 neurons. Scale bar = 100μm.

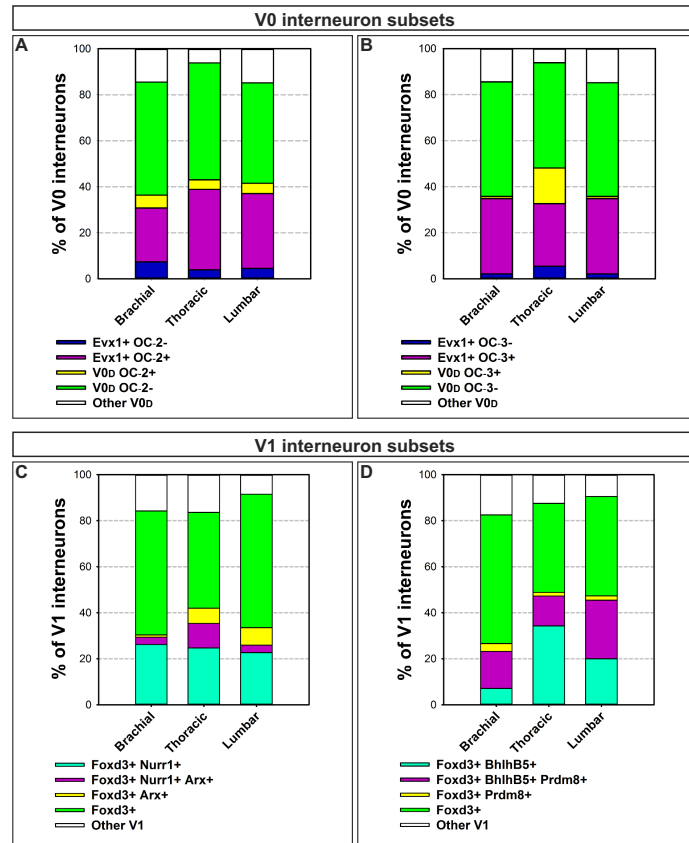


Figure S6. **Quantification of V0 or V1 subsets along the rostrocaudal axis of the spinal cord.** (A-D) Histograms of quantification of V0 and V1 interneuron subsets at brachial, thoracic and lumbar levels of the spinal cord at e12.5 show distinct distribution and proportion of different subsets within V0 and V1 interneuron cardinal class. Panels of 91 to 219 cells were counted for the V0 population, and 169 to 257 cells were counted for the V1 population, according to brachial, thoracic or lumbar levels. Histograms are normalized according to the total amount of cells in each ventral IN population, which corresponds to 100%. (n=3).





### **3.2. Onecut factors and Pou2f2 regulate diversification and migration of V2 interneurons in the developing spinal cord**

#### **3.2.1. Foreword**

The V2 populations migrate to a very precise position to adequately integrate the CPGs and to control the locomotor coordination. The type I V2a INs control the left-right alternation of the limbs, while type II V2a INs regulate the target reaching phase of the forelimb movements (Hayashi et al., 2018). The V2b INs, with V1 INs, control the alternation of flexor and extensor muscle contractions (Zhang et al., 2014). Finally, the V2d contribute to the generation of the locomotor rhythm (Dougherty et al., 2013). Their settling position seems to be a key determinant of their proper connectivity with other INs or MNs. Our previous study demonstrated that the V2 populations are differentially distributed along the antero-posterior axis of the spinal cord, while characterized subpopulations are distributed homogeneously (Francius et al., 2013). However, the mechanisms underlying the control of the V2 IN diversification and migration remain unknown.

In this study, we elucidate the role of the OC transcription factors during V2 IN diversification and migration. In order to study the consequences of the absence of OC factors on V2 IN distribution, we adapted a quantitative analysis of cell distribution within the developing spinal cord. The distribution of each V2 population was assessed by measuring the relative position of each cells on the medio-lateral (ML) and the dorso-ventral (DV) axes of the spinal cord. The length of the medio-lateral axis, corresponding to the hemicord width, was defined as the distance from the central canal to the lateral edge of the spinal cord, and the length of the dorso-ventral axis, corresponding to the spinal cord height, was

defined as the distance from the ventral limit of the central canal to the dorsal edge of the spinal cord. For each IN, the distance (D) and the angle ( $\alpha$ ) were measured from the ventral limit of the central canal to the IN soma. The ML and DV positions of each IN are the percentage of the spinal cord height (H) and hemicord width (W) measured as:  $(D_{IN} * \sin_{\alpha IN})/H$  and  $(D_{IN} * \cos_{\alpha IN})/W$ , respectively. The density graphs were obtained by the superposition of IN position from several sections ( $n \geq 5$ ) and from distinct embryos ( $n=3$ ).

Moreover, in order to identify molecular and cellular mechanisms downstream of OC factors controlling the V2 diversification and migration, we performed a microarray comparison of the transcriptome of 5 control and 5 OC-deficient spinal cords at e11.5, i.e. when a significant number of V2 INs are generated and when they are initiating their migration (GEO repository accession number: GSE117871). Probably due to a lack of sensitivity of the microarray experiment, we only identified few candidates downstream of the OC factors (table 6). We controlled the microarray results by either RT-qPCR or by *in situ* hybridization (ISH). Among genes showing a differential expression: some of them were not expressed in the ventral region of the spinal cord, others were false positives, or the tools (antibodies, transgenic mice) to further study some candidates were not available. In the absence of OC factors, Pou2f2 was 1.57-fold upregulated. Thus, we uncovered a regulator downstream of OC factors, Pou2f2, that contribute to the regulation of the V2 IN migration.

## Results

| Candidates                                               | Symbols | FC microarray | FC RT-qPCR | ISH |
|----------------------------------------------------------|---------|---------------|------------|-----|
| Polyamine modulated factor1 binding protein1             | Pmfbp1  | 0,28          | N.D        | ✓   |
| Pappalysin 2                                             | Pappa2  | 0,33          | 0.3        | ✓   |
| NK6 homeobox 3                                           | Nkx6.3  | 0,62          | N.D        | ✓   |
| Secreted frizzled-related sequence protein 5             | Sfrp5   | 0,69          | 0.83       | N.D |
| Dorsal inhibitory axon guidance protein                  | Draxin  | 0,7           | 1.42       | ✓   |
| Synuclein                                                | Sncg    | 0,7           | N.D        | N.D |
| NK6 homeobox 2                                           | Nkx6.2  | 0,7           | N.D        | ✓   |
| Stathmin-like 4                                          | Stmn4   | 0,71          | 1.14       | ✓   |
| Tubulin polymerization-promoting protein family member 3 | Tppp3   | 0,74          | 0.95       | N.D |
| Slowmo homolog 1                                         | Slmo1   | 1,29          | 1.43       | ✓   |
| POU domain class2, transcription factor 2                | Pou2f2  | 1,57          | See below  | ✓   |
| Kallikrein 1-related peptidase b22                       | Klk1b22 | 3,2           | 1.19       | ✓   |

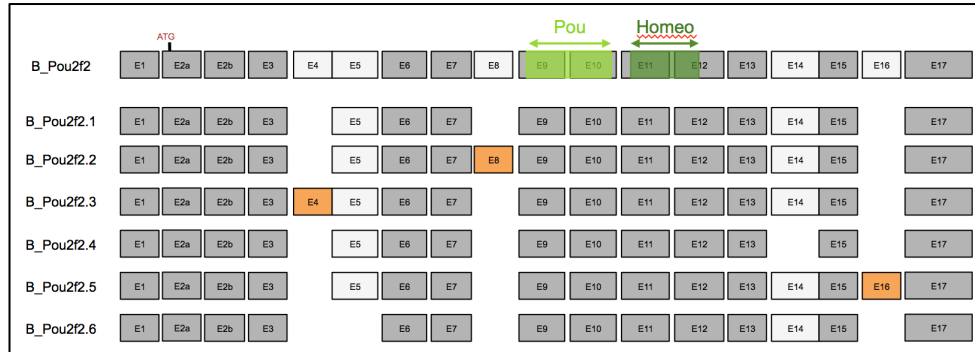
**Table 6: Candidates downstream of the OC factors.** The differential expression of the selected candidates between control and *Oc* mutant spinal cords obtained after the microarray experiments were next evaluate by RT-qPCR and their expression profile was characterized by ISH. FC = Fold change; N.D: not done.

### The Pou2f2 transcription factor

Pou2f2, also called Octamer protein-2 (Oct-2), is a POU family transcription factor characterized by a bipartite DNA-binding domain (the POU-domain) composed of a POU-specific and a POU-homeo domains separated by a short flexible linker. Both domains are required for interacting with DNA via helix-turn-helix motifs and bind to an octamer motif (ATGCAAAT or ATTTGCAT) with a high affinity. Pou2f2 does not dimerize, only a single molecule of Pou2f2 is required for binding to the octamer motif. In addition, it has been demonstrated in other protein, that the POU-specific serves also for protein-protein interactions (Ingraham et al., 1990; Kristie et al., 1989; Stern et al., 1989; Sturm and Herr, 1988). In addition to the octamer motif, Pou2f2 binds to the overlapping octamer/TAATGARAT motif with a higher affinity than either an octamer or an

octamer-related TAATGARAT motif alone (Clerc et al., 1988; Dent and Latchman, 1991; Stern et al., 1989; Sturm and Herr, 1988; Wirth et al., 1991). Pou2f2 is also characterized by 2 transactivation domains: The N-terminal glutamine-rich transactivation domain and the C-terminal serine-, threonine-, and proline-rich transactivation domain. The C-terminus is essential for the transactivating potential, while the N-terminus is not (Annweiler et al., 1992; Annweiler et al., 1994; Pfisterer et al., 1994).

Although a single gene on mouse chromosome 7 encodes Pou2f2, multiple isoforms are generated by alternative splicing: Pou2f2.1 to Pou2f2.6. These isoforms differ in their N- and C-terminal transactivation domains (Figure 30) (Wirth et al., 1991). In addition to the 6 described isoforms, a smaller form of Pou2f2, named Mini-Oct, exists. This form is transcriptionally neutral because it lacks the N- and C-terminal transactivation domains, and could serve to downregulate the transcriptional effect of the active octamer-binding factors (Stoykova et al., 1992). Finally, a seventh Pou2f2 isoform resulting from alternative splicing is expressed in the mammary gland. Pou2f2.7 has additional 22 amino acids close to the N-terminus and a novel 76 amino acid sequence at the C-terminus. Although it has an intact POU-domain, Pou2f2.7 is unable to bind to the octamer motif, unlike all the other known isoforms (Dong and Zhao, 2007).



**Figure 30: Sequences of the Pou2f2 isoforms resulting from alternative splicing in B cells (Wirth et al., 1991).** The 6 Pou2f2 isoforms present in the B cells (B\_Pou2f2.1 to B\_Pou2f2.6) are characterized by invariant exons (dark grey), alternative exons 4, 5, 8, 14 or 16 (light grey) and isoform-specific exons 4, 8 or 16 (orange). They contain a POU-specific domain (light green) encoded by exons 9 and 10 and a POU-type homeodomain (dark green) encoded by exons 11 and 12.

The Pou2f2 isoform transcriptional activity was assessed by co-transfection of expression vectors for different Pou2f2 isoforms and plasmids containing the chloramphenicol acetyltransferase gene downstream of an octamer-containing promoter. Hence, Pou2f2.1, Pou2f2.2 and Pou2f2.3 are activators, while Pou2f2.4 and Pou2f2.5 are inhibitors. This difference in their transcriptional activity can be explained by the fact that the 6 isoforms differ from each other in the transactivation domains (Annweiler et al., 1994; Camos et al., 2014; Hatzopoulos et al., 1990; Lillycrop and Latchman, 1992; Wirth et al., 1991). Pou2f2.1 appears to be predominant in B cells, while Pou2f2.4 and Pou2f2.5 are the main isoforms in neuronal cell lines (Liu et al., 1995). Neuronal Pou2f2 isoforms act as repressors either by directly binding to the octamer motif or by preventing the activation mediated by Oct-1, their homolog, by binding to adjacent or overlapping DNA binding sites (Dent et al., 1991).

Pou2f2 is expressed in B lymphocytes and in neuronal cell lines. In the central nervous system, Pou2f2 was detected in the developing diencephalon, mesencephalon, metencephalon, myelencephalon, neural tube, and dorsal root ganglia by *in situ* hybridization. It is also strongly expressed in the mouse adult

brain (Camos et al., 2014; Hatzopoulos et al., 1990; Lillycrop and Latchman, 1992; Schreiber et al., 1990; Stoykova et al., 1992; Wood et al., 1992). The *Pou2f2* mutant mice develop normally but die at birth from undetermined reasons. Nevertheless, Lynn M. Corcoran et al., 2004 highlighted that the C-terminal transactivation domain is required for postnatal survival by using *Pou2f2* mutant mice in which the C-terminal domain was truncated. In B cells, *Pou2f2* is not required for B lymphocytes production but for their maturation into immunoglobulin-secreting B cells and their ability to respond positively to a B cell receptor signal (Corcoran et al., 1993; Corcoran et al., 2004). In neuronal cells, *Pou2f2* can act as a bifunctional regulator of neuronal differentiation. Indeed, *Pou2f2.4*, which lacks the C-terminal transactivation domain, inhibits the neuronal differentiation of embryonic stem cells, unlike *Pou2f2.2* that is able to activate this process (Theodorou et al., 2009).

The paper is submitted to Development. In this work, I carried out all the experiments except the characterization of OC factor expression profile in the different subpopulations within each ventral IN population (realized by Collin A., who I did supervise during her Master Thesis).

**Onecut factors and Pou2f2 regulate diversification and migration of V2 interneurons in the mouse developing spinal cord**

Audrey Harris<sup>1</sup>, Gauhar Masgutova<sup>1</sup>, Amandine Collin<sup>1</sup>, Mathilde Toch<sup>1</sup>, Maria Hidalgo-Figueroa<sup>1,4</sup>, Benvenuto Jacob<sup>2</sup>, Lynn M. Corcoran<sup>3</sup>, Cédric Francius<sup>1,5</sup> and Frédéric Clotman<sup>1\*</sup>

<sup>1</sup> Université catholique de Louvain, Institute of Neuroscience, Laboratory of Neural Differentiation, Brussels, Belgium; <sup>2</sup> Université catholique de Louvain, Institute of Neuroscience, System and Cognition division, Brussels, Belgium; <sup>3</sup> The Walter and Eliza Hall Institute, Molecular Immunology Division and Immunology Division, Parkville, Victoria, Australia.

**For correspondence** : frederic.clotman@uclouvain.be

<sup>4</sup> Present address : Neuropsychopharmacology & Psychobiology Research Group, Area of Psychobiology, Department of Psychology, University of Cadiz, Spain; Instituto de Investigación e Innovación en Ciencias Biomédicas de Cádiz (INIBICA), Spain

<sup>5</sup> Present address : PAREXEL International, France



### Abstract

Acquisition of proper neuronal identity and position is critical for the formation of neural circuits. In the embryonic spinal cord, cardinal populations of interneurons diversify into specialized subsets and migrate to defined locations within the spinal parenchyma. However, the factors that control interneuron diversification and migration remain poorly characterized. Here, we show that the *Onecut* transcription factors are necessary for proper diversification and migration of the spinal V2 interneurons in the developing spinal cord. Furthermore, we uncover that these proteins restrict and moderate the expression of spinal isoforms of *Pou2f2*, a transcription factor known to regulate B-cell differentiation. By gain- or loss-of-function experiments, we show that *Pou2f2* contribute to regulate the distribution of V2 populations in the developing spinal cord. Thus, we uncovered a genetic pathway that regulates the diversification and the migration of V2 interneurons during embryonic development.

### Significance statement

In this study, we identify genetic regulators of spinal interneuron diversification and migration, two events that are critical for proper CNS development. We also highlight how the use of different promoters and alternative splicing contribute to the recruitment of the same transcriptional regulator in completely different processes, namely B-cell maturation and spinal interneuron migration. This expands our current knowledge about the development of neural circuits that regulate critical behaviors in animal or human species, including walking, running and swimming. Finally, it constitutes bases for the identification of the molecular mechanisms involved in these processes, for the evaluation of similar mechanisms after adult spinal cord injury, and to characterize and control the *in vitro* differentiation of stem/progenitor cells into spinal interneuron.



### **3.2.2. Introduction**

Neuronal migration is a critical feature of CNS development. It enables neurons, which initiate differentiation in the vicinity of the ventricular zone wherein neural progenitors are located, to reach adequate location in the nervous parenchyma and properly integrate into neural circuits. The mechanisms that regulate neuronal migration have been extensively studied in the developing brain, particularly for cortical interneurons (INs) that are produced in the ganglionic eminences and undergo a long tangential displacement to invade the cortex and integrate into cortical circuits (Barber and Pierani, 2016; Guo and Anton, 2014). In contrast, the factors that control IN migration in the developing spinal cord remain almost totally unknown.

In the embryonic spinal cord, distinct neuronal populations are generated from different progenitor domains orderly distributed along the dorsoventral axis of the ventricular zone. These progenitors produce motor neurons and multiple populations of ventral or dorsal INs (Lai et al., 2016; Lu et al., 2015). In contrast to motor neurons, spinal IN populations do not organize into columns along the anteroposterior axis of the spinal cord (Francius et al., 2013). Nevertheless, they each migrate according to a stereotyped pattern and settle down at specific focused or diffuse locations in the spinal parenchyma (Grossmann et al., 2010b). Recent studies demonstrated that proper neuronal distribution is critical for adequate formation of spinal circuits. Indeed, the clustering and dorsoventral settling position of motor neuron pools critically pattern sensory input specificity (Surmeli et al., 2011). Position of dorsal INs along the mediolateral axis in lamina V determines their connectivity with sensory afferents (Hilde et al., 2016) while extensor and flexor premotor INs segregate along the medio-lateral axis of the spinal cord (Tripodi et al., 2011). Positional distinctions among premotor INs additionally correlate with their output to different motor columns (Goetz et al.,

2015) and differential distribution of V1 IN subsets constrain patterns of input from sensory and from motor neurons (Bikoff et al., 2016). Consistently, distinct ventral IN subsets are differentially distributed along the anteroposterior axis of the spinal cord (Francius et al., 2013; Hayashi et al., 2018) and integrate into specific local microcircuit modules (Hayashi et al., 2018). However, the molecular mechanisms that regulate proper distribution of spinal INs remain elusive.

During their migration, cardinal populations of spinal neurons undergo progressive diversification into distinct subsets that exert specific functions in spinal circuits (Catela et al., 2015; Lai et al., 2016; Lu et al., 2015). For example, V2 INs diversify into major V2a and V2b and minor V2c and V2d populations. V2a and V2d are excitatory neurons that participate in left-right alternation at high speed and contribute to rhythmic activation of locomotor circuits, respectively (Crone et al., 2008; Dougherty and Kiehn, 2010; Dougherty et al., 2013). V2b cells are inhibitory INs that participate in alternation of flexor vs extensor muscle contraction (Britz et al., 2015). As observed for V1 INs (Bikoff et al., 2016), V2 cells further diversify into more discrete subpopulations differentially distributed along the anteroposterior axis of the spinal cord (Francius et al., 2013; Hayashi et al., 2018). However, specific functions of these IN subsets have not been investigated yet, and the mechanisms that govern their production remain currently unknown. Recently, we identified Onecut (OC) transcription factors as regulators of neuronal diversification (Francius and Clotman, 2014; Kabayiza et al., 2017; Roy et al., 2012) and of dorsal IN migration (Kabayiza et al., 2017) in the developing spinal cord. OC factors, namely Hepatocyte Nuclear Factor-6 (HNF-6, or OC-1), OC-2 and OC-3, are transcriptional activators present in the digestive tract and in the CNS during embryonic development (Jacquemin et al., 1999; Jacquemin et al., 2003b; Landry et al., 1997; Lemaigre et al., 1996; Vanhorenbeeck et al., 2002). In neural tissue, they regulate production (Espana and Clotman, 2012a), diversification (Francius

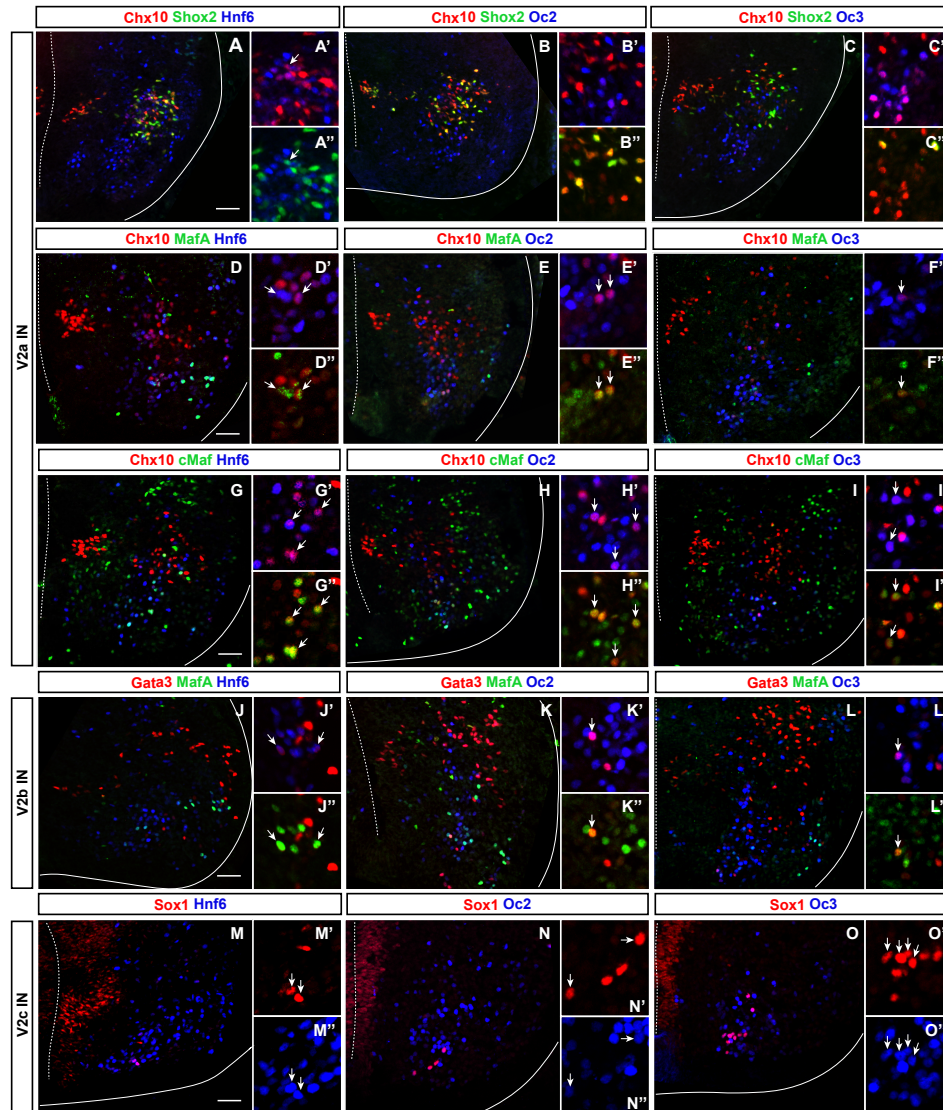
and Clotman, 2014; Kabayiza et al., 2017; Roy et al., 2012), distribution (Audouard et al., 2013; Espana and Clotman, 2012a, b; Kabayiza et al., 2017) and maintenance (Espana and Clotman, 2012a, b; Stam et al., 2012) of specific neuronal populations, as well as the formation of neuromuscular junctions (Audouard et al., 2012). Here, we demonstrate that OC factors regulate the diversification and the distribution of V2 INs during spinal cord development. Analyses of OC-deficient embryos showed defective production of specific subpopulations of V2a INs, as well as abnormal distribution of V2a and V2b cells in the developing spinal cord. Furthermore, we uncovered that OC proteins act upstream of specific spinal isoforms of Pou2f2, a POU family transcription factor. Using gain- or loss-of-function experiments, we demonstrated that, as observed for OC factors, Pou2f2 regulates the distribution of V2 INs in the developing spinal cord. Thus, we uncovered a genetic pathway that regulates the diversification and the migration of V2 INs during embryonic development.

### **3.2.3. Results**

#### **OC factors are present in multiple subsets of spinal V2 interneurons**

In the developing spinal cord, OC factors contribute to diversification, migration and maintenance of different neuronal populations (Kabayiza et al., 2017; Roy et al., 2012; Stam et al., 2012). To study V2 interneuron diversification, we previously established a repertoire of markers that divide embryonic V2 cells into multiple subpopulations (Francius et al., 2013). Although OC factors have been detected in V2 interneurons (Francius and Clotman, 2010), their distribution in V2 subsets has not been investigated yet. Therefore, we first determined the presence of each OC in these V2 subpopulations at e12.5.

V2a interneurons include neuronal subsets characterized by the presence of Shox2, MafA, cMaf, Bhlhb5 or Prdm8 (Francius et al., 2013). Only Hnf6 was detected in few Shox2<sup>+</sup> V2a cells (Figure 31A-C"; Table 7). In contrast, the 3 OC proteins were detected in MafA<sup>+</sup> and cMaf<sup>+</sup> V2a subpopulations (Figure 31D-I"; Table 7), but not in Bhlhb5<sup>+</sup> or Prdm8<sup>+</sup> cells (Table 7; data not shown). V2b interneurons include similar subsets except for Shox2<sup>+</sup> and cMaf<sup>+</sup> cells, and contain an additional MafB<sup>+</sup> subpopulation (Francius et al., 2013). OC were present in MafA<sup>+</sup> but not in MafB<sup>+</sup>, Bhlhb5<sup>+</sup> or Prdm8<sup>+</sup> V2b subsets (Figure 31J-L"; Table 7; data not shown). In addition, OC were detected in V2c (non-progenitor Sox1<sup>+</sup> cells; Figure 31M-O"; Table 7) but not in V2d (Shox2<sup>+</sup>Chx10<sup>-</sup>) cells (Figure 31A-C"; Table 7). Thus, OC factors are present in multiple subpopulations of V2 interneurons.



**Figure 31: OC factors are present in multiple subsets of V2 interneurons. (A-I'')** Immunolabelings for OC, the V2a generic marker Chx10 and markers of V2a subpopulations (Francius et al., 2013) on transverse spinal cord sections (brachial or thoracic levels) of e12.5 wild-type mouse embryos. In each figure, the right ventral quadrant of the spinal cord is shown. Only HNF-6 is detected in Shox2+ V2a cells (arrow in A-C''), whereas the 3 OC are present in the MafA+ and in the cMaf+ V2a subsets (arrows in D-I''). **(J-L'')** Immunolabelings for OC, the V2b generic marker Gata3 and MafA. The 3 OC proteins are detected in MafA+ V2b interneurons (arrows). **(M-O'')** Immunolabelings for OC and the V2c marker Sox1 demonstrate that OC factors are present in a majority of V2c interneurons (arrows). Sox1 in the ventricular zone labels neural progenitors. Scale bar = 50  $\mu$ m.



| V2 – e12.5  |                |       |      |      |
|-------------|----------------|-------|------|------|
| Populations | Subpopulations | HNF-6 | OC-2 | OC-3 |
| V2a – Chx10 |                | +     | +    | +    |
|             | Shox2          | +     | -    | -    |
|             | MafA           | +     | +    | +    |
|             | cMaf           | +     | +    | +    |
|             | Bhlhb5         | -     | -    | -    |
|             | Prdm8          | -     | -    | -    |
| V2b – Gata3 |                | +     | +    | +    |
|             | MafA           | +     | +    | +    |
|             | MafB           | -     | -    | -    |
|             | Bhlhb5         | -     | -    | -    |
|             | Prdm8          | -     | -    | -    |
| V2c – Sox1  |                | +     | +    | +    |
| V2d – Shox2 |                | -     | -    | -    |

**Table 7: OC factors are present in specific populations and subpopulations of V2 interneurons.** The V2 populations, including V2a, V2b, V2c and V2d, are subdivided in smaller subpopulations characterized by differential expression of transcription factors (Francius et al., 2013). OC factors are detected in specific populations and subpopulations of V2 interneurons.

### OC factors regulate the diversification of spinal V2 interneurons

To determine whether OC proteins contribute to the development of V2 interneuron subsets, we characterized the phenotype of these cells in *Hnf6/Oc2* double-mutant embryos, which lack the three OC factors in the developing spinal cord (Kabayiza et al., 2017; Roy et al., 2012). Given that the number and the distribution of cells in each interneuron subpopulation vary along the anteroposterior axis of the spinal cord (Francius et al., 2013; Hayashi et al., 2018; Sweeney et al., 2018), this analysis was systematically performed at brachial, thoracic and lumbar levels at e12.5 and e14.5.

In the absence of OC factors, the total number of Chx10+ interneurons was not significantly changed (Figure 32A-D; Supplementary Figure S7A-B), although a

trend toward reduction was detected at brachial level at e12.5 (Figure 32C). A large subset of V2a cells contains Shox2 (Figure 31A-C"; 2E-F"; (Dougherty et al., 2013)). Consistently, the number of Chx10+Shox2+ interneurons was not changed in the absence of OC proteins (Figure 32E-H; Supplementary Figure S7C-D"). These observations suggest that OC are not necessary for V2a interneuron production. In contrast, the smaller V2a subpopulations wherein OC factors were detected in control embryos, characterized by the presence of MafA (Figure 31D-F") or cMaf (Figure 31G-I"), were almost completely lost in OC mutant embryos (Figure 32I-P; Supplementary Figure S7E-H"). As the total number of Chx10+ and of Shox2+ V2a was not changed (Figure 32A-H; Supplementary Figure S7A-D"), the loss of the MafA+ or cMaf+ subsets may be compensated for by expansion of other V2a subpopulations. However, we were unable to detect expansion of any alternative V2a subset (data not shown), suggesting that the absence of OC proteins favor differentiation of V2a subpopulations that remain to be identified. Nevertheless, our data indicate that OC factors are required either for the expression of V2 subpopulation markers or for the differentiation of specific V2a interneuron subsets.

To discriminate between these possibilities and to evaluate the contribution of OC factors to V2b diversification, we characterized the phenotype of V2b interneurons and of their MafA+ subpopulation in the absence of OC proteins. As observed for V2a interneurons, the total number of V2b cells was not changed in OC mutant embryos (Figure 32Q-T; Supplementary Figure S7I-J), although a trend toward reduction was observed at brachial level at e12.5 (Figure 32S). As for V2a, OC are present in the MafA+ V2b subpopulation (Figure 31J-L"). However, in contrast to V2a, the MafA+ V2b interneurons were present in normal number in OC mutant embryos (Figure 32U-X; Supplementary Figure S7K-L"). Hence, OC factors are not necessary for *MafA* expression in spinal V2 interneurons nor for

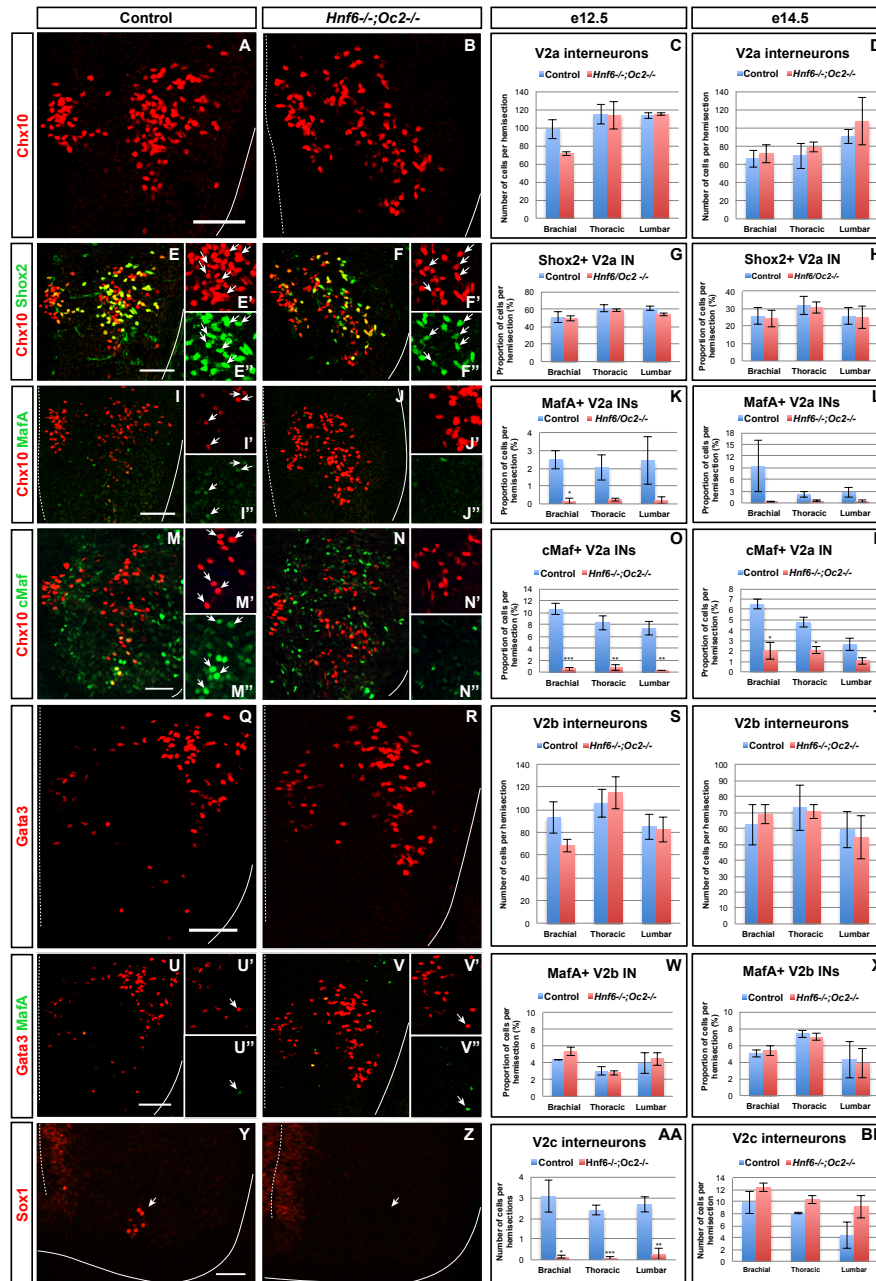
the production of the MafA<sup>+</sup> V2b subset, although they are required for the differentiation of the MafA<sup>+</sup> and of the cMaf<sup>+</sup> V2a subpopulations.

Finally, we assessed the requirement for OC in the production of V2c interneurons, a V2 population strongly related to V2b cells (Panayi et al., 2010) wherein OC are broadly present (Figure 31M-O"). Although weak production of Sox1 in spinal progenitors was preserved, V2c cells characterized by high Sox1 levels were scarcely detectable in OC mutant embryos at e12.5 (arrows in Figure 32Y-AA). However, the number of V2c was normal at e14.5 (Figure 32BB; Supplementary Figure S7M-N), suggesting that the absence of OC delays the differentiation of V2c interneurons without affecting the V2b population. Taken together, these observations demonstrate that OC proteins are not required for V2 interneuron production but regulate the diversification of V2 interneurons into multiple subpopulations.

---

Shox2<sup>+</sup> V2a is affected neither at e12.5 (**E-G**) nor at e14.5 (**H**). In contrast, quantitative analysis of control or *Hnf6*<sup>-/-</sup>; *Oc2*<sup>-/-</sup> littermates at e12.5 (**I-K**) and at e14.5 (**L**) shows reduction in MafA<sup>+</sup> V2a interneurons in double mutants as compared to control embryos. Similarly, the number of cMaf<sup>+</sup> V2a interneurons is significantly reduced at e12.5 (**M-O**) and e14.5 (**P**) in the absence of OC factors. At e12.5 (**Q-S**) and e14.5 (**T**), the production of the V2b interneurons is not affected in *Hnf6*<sup>-/-</sup>; *Oc2*<sup>-/-</sup> embryos. The generation of the MafA<sup>+</sup> V2b interneurons is also unchanged at e12.5 (**U-W**) or e14.5 (**X**). At e12.5 (**Y-AA**), the number of V2c interneurons is dramatically reduced in the absence of OC factors (Sox1 in the ventricular zone labels neural progenitors). However, this is no longer the case at e14.5 (**BB**). Mean values ± SEM. \* p≤0.05 ; \*\* p≤0.01 ; \*\*\* p≤0.001. Scale bar = 50 μm.

## Results



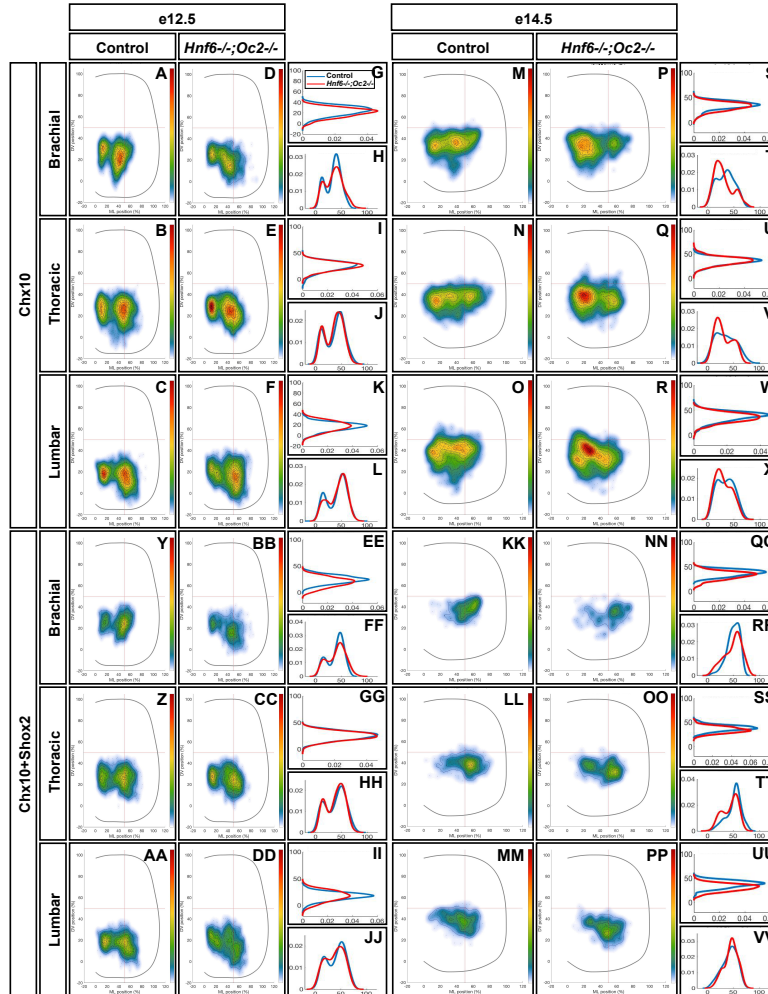
**Figure 32: OC factors regulate the diversification of the V2 interneurons.** Immunolabelings on transverse spinal cord sections (brachial or thoracic levels) of control or *Hnf6*<sup>-/-</sup>;*Oc2*<sup>-/-</sup> double-mutant embryos. At e12.5 (A-C) and e14.5 (D), the production of the V2a Chx10+ interneurons is not altered in the absence of OC factors. Similarly, the number of

**OC factors regulate the distribution of spinal V2 interneurons**

Although the total number of V2a or V2b interneurons was not affected by the absence of OC factors, careful examination of immunofluorescence labelings suggested that, as observed for spinal dorsal interneurons (Kabayiza et al., 2017), OC proteins may regulate the distribution of V2 interneurons in the developing spinal cord (Figure 32A-B; Q-R). Therefore, quantitative distribution analyses (Kabayiza et al., 2017) were performed for each V2 population present in OC mutant embryos at brachial, thoracic or lumbar levels at e12.5, namely in the course of ventral interneuron migration, and at e14.5, i.e. when ventral interneuron migration in the transverse plane of the spinal cord is completed.

At e12.5 in control embryos, V2a interneurons distributed in 2 connected clusters, a major central group and a minor medial group, at each level of the spinal cord. In mutant embryos, V2a cells similarly distributed in connected central and medial groups. However, the relative cell distribution between the 2 clusters seemed altered, with less central cells at brachial level and less medial cells at lumbar levels (Figure 33A-L). Altered V2a distribution on the mediolateral axis was confirmed at e14.5. In control embryos, the 2 V2a groups did coalesce in a more evenly-distributed population that occupied ~70% of the medio-lateral axis. In mutant embryos, V2a interneurons remained segregated into 2 distinct, although connected, clusters with a majority of cells in medial position (Figure 33M-X). Thus, absence of OC factors perturbs proper distribution of the V2a interneurons and restricts at e14.5 migration of a fraction of V2a cells in a medial position. To assess whether these distribution defects may only affect V2a subsets or impact the whole V2a population, we sought to characterize the distribution of V2a subpopulations. However, the MafA<sup>+</sup> and cMaf<sup>+</sup> subsets were absent in OC mutants. Therefore, only the broader Shox2<sup>+</sup> subpopulation was analyzed. Similar

observations were made as for the whole V2a population (Figure 33Y-VV), confirming that absence of OC factors alters the distribution of V2a interneurons.

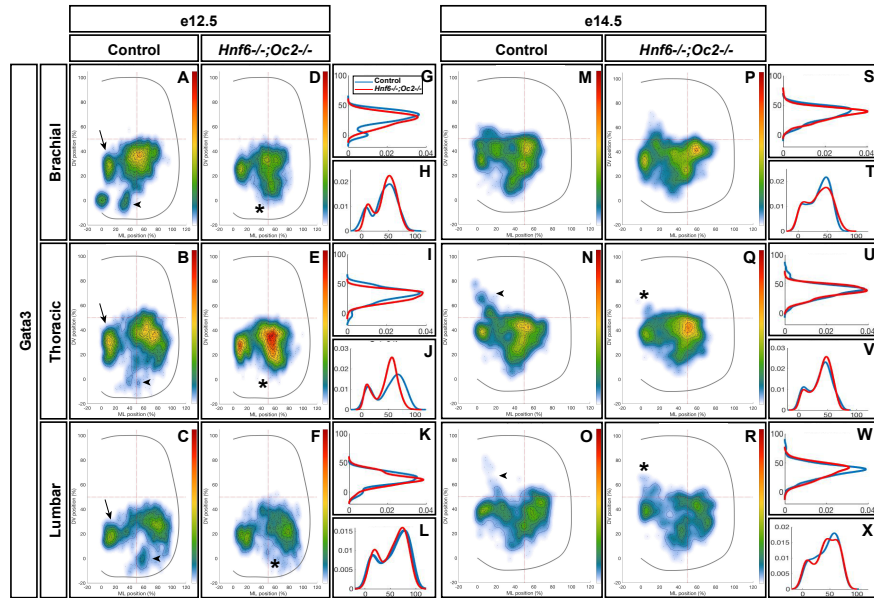


**Figure 33: OC factors regulate the distribution of V2a interneurons.** Distribution of V2a interneurons on transverse section of the spinal cord in control or *Hnf6*<sup>-/-</sup>;*Oc2*<sup>-/-</sup> double-mutant embryos at brachial, thoracic or lumbar level (only the right hemisection is shown). Two-dimension distribution graphs (left) show integration of cell distribution from multiple sections of multiple embryos of each genotype. One-dimension graphs (right) compare density distribution in control (blue) and in double-mutant embryos (red) on the dorso-ventral (upper) or the medio-lateral (lower) axis of the spinal cord (see Materials and methods for details). (A-C) At e12.5 in control embryos, V2a interneurons distribute in 2 connected clusters, a major central group and a minor medial group, at each level of the spinal cord. (D-L) In mutant embryos, the relative cell →

To assess whether OC also regulate the position of other V2 populations, we studied the distribution of V2b interneurons. At e12.5 in control embryos, V2b cells distributed in a major central (brachial level) or lateral (thoracic and lumbar levels) cluster with minor subsets located more medially (arrows in Figure 34A-L) or ventrally (arrowheads in Figure 34A-L). In OC mutant embryos at e12.5, the major population was more compact, more centrally located and slightly more ventral. In addition, the ventral V2b subset was significantly depleted (asterisks in Figure 34A-L). Consistently, at e14.5, V2b interneurons in the central cluster remained significantly more compact at thoracic level in the absence of OC factors, and identical trends were observed at brachial and lumbar levels (Figure 34M-X). In addition, a small contingent of V2b migrating towards the medio-dorsal spinal cord in control embryos (arrowheads in Figure 34M-X) was missing in OC mutant littermates (asterisks in Figure 34M-X). Thus, as observed for V2a cells, absence of OC factors perturbs proper distribution of the V2b interneurons. The MafA<sup>+</sup> V2b subset was too small to enable reliable distribution analysis. Taken together, these observations demonstrate that, in addition to V2 diversification, the OC transcription factors regulate proper distribution of V2 interneurons during spinal cord development.

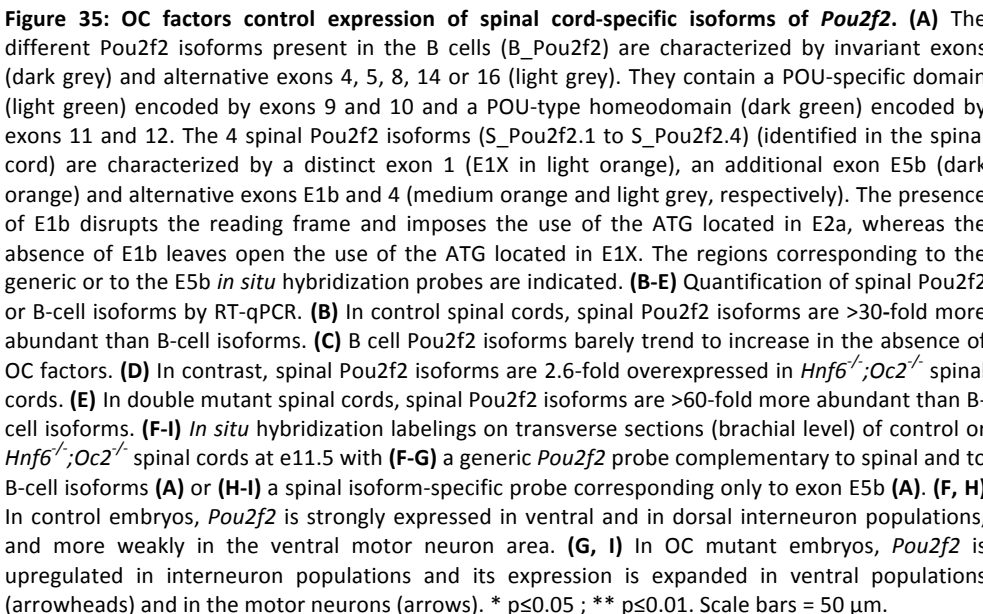
---

distribution between the 2 clusters seems altered, with relatively less central cells at brachial level and less medial cells at lumbar levels (n=3, asterisks; n=3,  $p \leq 0.001$  at brachial and lumbar levels;  $p=0.1703$  at thoracic level). **(M-X)** Altered V2a distribution on the mediolateral axis is confirmed at e14.5. **(M-O, S-X)** In control embryos, the 2 V2a groups coalesce in a more evenly-distributed population that occupied ~70% of the medio-lateral axis. **(P-X)** In mutant embryos, V2a interneurons remain segregated into 2 distinct, although connected, clusters with a majority of cells in medial position (n=3,  $p \leq 0.001$ ). **(Y-VV)** Similar observations are made for the Shox2<sup>+</sup> V2a subpopulation. At e12.5 in control embryos **(Y-AA)**, Shox2<sup>+</sup> V2a distribute in a minor medial and a major lateral cluster. **(BB-JJ)** In mutant embryos, these cells are slightly more ventral at brachial level and distribute more evenly between the two clusters at brachial and lumbar levels (n=3,  $p \leq 0.001$  at brachial and lumbar levels;  $p=0.077$  at level thoracic level). **(KK-VV)** At e14.5, Shox2<sup>+</sup> V2a in the mutant were slightly more ventral and remained more medial than in control embryos (n=3,  $p \leq 0.001$ ).



**Figure 34: OC factors regulate the distribution of V2b interneurons.** (A-C) At e12.5 in control embryos, V2b cells are distributed in a major central (brachial level) or lateral (thoracic and lumbar levels) cluster with minor subsets located more medially (arrows) or ventrally (arrowheads). (D-L) In OC mutant embryos at e12.5, the major population remains more compact, more centrally located and slightly more ventral. In addition, the ventral V2b subset is significantly depleted (asterisks;  $n=3$ ,  $p \leq 0.001$  at brachial and thoracic levels;  $p=0.3135$  at lumbar level). (M-X) Consistently, at e14.5, V2b interneurons in the central cluster are more compact at thoracic level in the absence of OC factors, and identical trends were observed at brachial and lumbar levels, and a small contingent of V2b migrating towards the medio-dorsal spinal cord in control embryos (arrowheads) is missing in OC mutant littermates (asterisks;  $n=3$ ,  $p \leq 0.001$  at thoracic level;  $p=0.3127$  and  $0.7999$  at brachial and lumbar levels, respectively).





**OC factors control expression of neuronal-specific isoforms of *Pou2f2***

In an effort to identify genes downstream of OC that may contribute to V2 interneuron differentiation and distribution, we performed a microarray comparison of control and of OC-deficient spinal cord transcriptome at e11.5, namely at the stage when significant numbers of V2 cells have been generated and are initiating migration. Among genes showing a differential expression level in the OC mutant spinal cord, *Pou2f2* was significantly upregulated (1.57-fold increase). *Pou2f2* (previously named Oct-2) is a transcription factor containing a POU-specific domain and a POU-type homeodomain (Figure 35A) that binds an octamer motif (consensus sequence ATGCAAAT) (Latchman, 1996). *Pou2f2* expression has been detected in B lymphocytes, in neuronal cell lines and in neural tissues including the developing CNS (Camos et al., 2014; Hatzopoulos et al., 1990; Lillycrop and Latchman, 1992). *Pou2f2* is required for differentiation of B lymphocytes and for postnatal survival (Corcoran et al., 1993; Corcoran et al., 2004; Hodson et al., 2016; Konig et al., 1995), and is able to modulate neuronal differentiation of ES cells (Theodorou et al., 2009). However, its role in the developing spinal cord remains unknown.

Based on work in B cells, multiple *Pou2f2* isoforms generated by alternative splicing have been described (Figure 35A; (Hatzopoulos et al., 1990; Lillycrop and Latchman, 1992; Liu et al., 1995; Stoykova et al., 1992; Wirth et al., 1991)). Therefore, we first determined whether similar isoforms are found in the developing spinal cord. However, using RT-PCR experiments, we systematically failed to obtain PCR products using upstream primers in described exon 1 (asterisks in Supplementary Figure S8A-B and data not shown; Table 8) and amplifications encompassing exons 5 to 6 generated predominant amplicons larger than expected (arrowheads in Supplementary Figure S8B; Table 8),

## Results

suggesting the existence of alternative exons in *Pou2f2* embryonic spinal cord transcripts (Figure 35A). Data mining the NCBI

| Amplification | Expected sizes             | B cells                    | Control spinal cord                      |
|---------------|----------------------------|----------------------------|------------------------------------------|
| E1 → E6       | 272 bp<br>390 bp<br>456 bp | 272 bp<br>390 bp<br>456 bp | No amplification                         |
| E7 → E9       | 174 bp<br>222 bp           | 174 bp<br>222 bp           | 174 bp                                   |
| E10 → E12     | 452 bp                     | 452 bp                     | 452 bp                                   |
| E13 → E17     | 160 bp<br>296 bp<br>370 bp | 160 bp<br>296 bp<br>370 bp | 370 bp                                   |
| E1 → E7       | 348bp<br>466 bp<br>532 bp  | 466 bp<br>532 bp           | No amplification                         |
| E2 → E7       | 284 bp<br>402 bp<br>468 bp | 402 bp<br>468 bp           | ~ 680 bp<br>~ 750 bp                     |
| E3 → E6       | 154 bp<br>272 bp<br>378 bp | 154 bp<br>272 bp<br>378 bp | 272 bp<br>378 bp<br>~ 550 bp<br>~ 600 bp |
| E4 → E7       | 340 bp                     | 340 bp                     | ~ 600 bp                                 |
| E5 → E7       | 201 bp                     | 201 bp                     | 201 bp<br>~ 460 bp                       |
| E6 → E7       | 160 bp                     | 160 bp                     | 160 bp                                   |
| E1X → E3      | 152 bp                     | No amplification           | 152 bp<br>~ 220 bp                       |
| E1X → E5b     | 554 bp<br>620 bp           | N.D                        | 554 bp<br>620 bp                         |
| E5b           | 234 bp                     | N.D                        | 234 bp                                   |
| E3 → E5b      | 429 bp<br>495 bp           | N.D                        | 429 bp<br>495 bp                         |
| E5b → E7      | 416 bp                     | N.D                        | 416 bp                                   |

**Table 8: *Pou2f2* isoforms in the developing spinal cord are different from B-cell isoforms.** Regions covering B lymphocytes or predicted *Pou2f2* isoforms (exons E1 or E1X to E17) were amplified by RT-PCR from B lymphocyte or embryonic spinal cord RNA. Orange cells = unexpected results.

Nucleotide database for *Pou2f2* sequences identified a predicted murine *Pou2f2* isoforms (X6 sequence, accession number XM\_006539651.3) with a different exon 1 (E1X) and an additional sequence between exons 5 and 6, the size of which (279 bp) corresponded to the size differences estimated in our amplifications encompassing exons 5 to 6 (arrowheads in Supplementary Figure S8B; Table 8). Using PCR primers in this predicted sequence, we were able to amplify a 5' region of *Pou2f2* from the alternative E1X exon and an additional sequence between exons 5 and 6 (Supplementary Figure S8C; Table 8), suggesting that alternative isoforms similar to this predicted sequence are produced in the developing spinal cord. However, amplifications from E1X systematically produced 2 amplicons (arrowheads in Supplementary Figure S8C; Table 8), suggesting the existence of an alternative exon downstream to E1X. Sequencing of our PCR products and alignment to genomic DNA confirmed that predominant *Pou2f2* isoforms in the developing spinal cord contain the alternative E1X exon, an additional exon (E5b) between exons 5 and 6, and can undergo alternative splicing of a short (61bp) exon (E1b) between E1X and exon 2 (Supplementary Figure S8D). E5b exon maintains the reading frame. In contrast E1b exon disrupts the reading frame, imposing the use of the ATG located in exon 2 to generate a functional *Pou2f2* protein, whereas the absence of E1b leaves open the use of an alternative upstream ATG located at the 3' end of E1X (Figure 35A; Supplementary Figure S8D). Hence, taking into account our RT-PCR and sequencing data, 4 neuronal *Pou2f2* isoforms that are different from the previously described B-cell or neural isoforms can be produced in the developing spinal cord (Fig 35A).

However, our RT-PCR experiments suggested that minor transcripts corresponding to B-cell isoforms are also present in the embryonic spinal cord (Supplementary Figure S8A-B; Table 8). To assess the relative abundance of each transcript type in this tissue and to evaluate the extent of their relative

overexpression in the absence of OC factors, we quantified each isoform type in control and in OC-deficient spinal cord at e11.5. In control spinal cords, spinal *Pou2f2* isoforms were >30-fold more abundant than B-cell isoforms (Figure 35B), consistent with our RT-PCR observations (Supplementary Figure S8). In the absence of OC factors, spinal isoforms were ~2.6-fold overexpressed whereas B-cell isoforms barely trended to increase (Figure 35C-D), resulting in spinal isoforms being >60-fold more abundant than B-cell transcripts (Figure 35E). Thus, OC factors repress expression of spinal *Pou2f2* isoforms in the developing spinal cord.

To confirm these data and to determine the expression pattern of *Pou2f2* in the ventral spinal cord, *in situ* hybridization was performed on sections from control or *Hnf6/Oc2* double-mutant spinal cords using either a generic *Pou2f2* probe complementary to spinal and to B-cell isoforms or a spinal isoform-specific probe corresponding only to exon E5b (Figure 35A). Using the generic *Pou2f2* probe on control tissue, we detected *Pou2f2* transcripts in the ventral region of the spinal cord, with lower expression levels in the location of the motor columns (arrows in Figure 35F). In OC mutant embryos, *Pou2f2* expression was globally increased and additionally expanded in the ventral area (arrowheads in Figure 35F-G) including the motor neuron territories (arrows in Figure 35F-G). Similar observations were made with the spinal-specific probe, although the signal was much weaker probably owing to the shorter length of the probe (Figure 35H-I). Thus, OC factors restrict and moderate *Pou2f2* expression in ventral spinal populations likely including V2 interneurons.

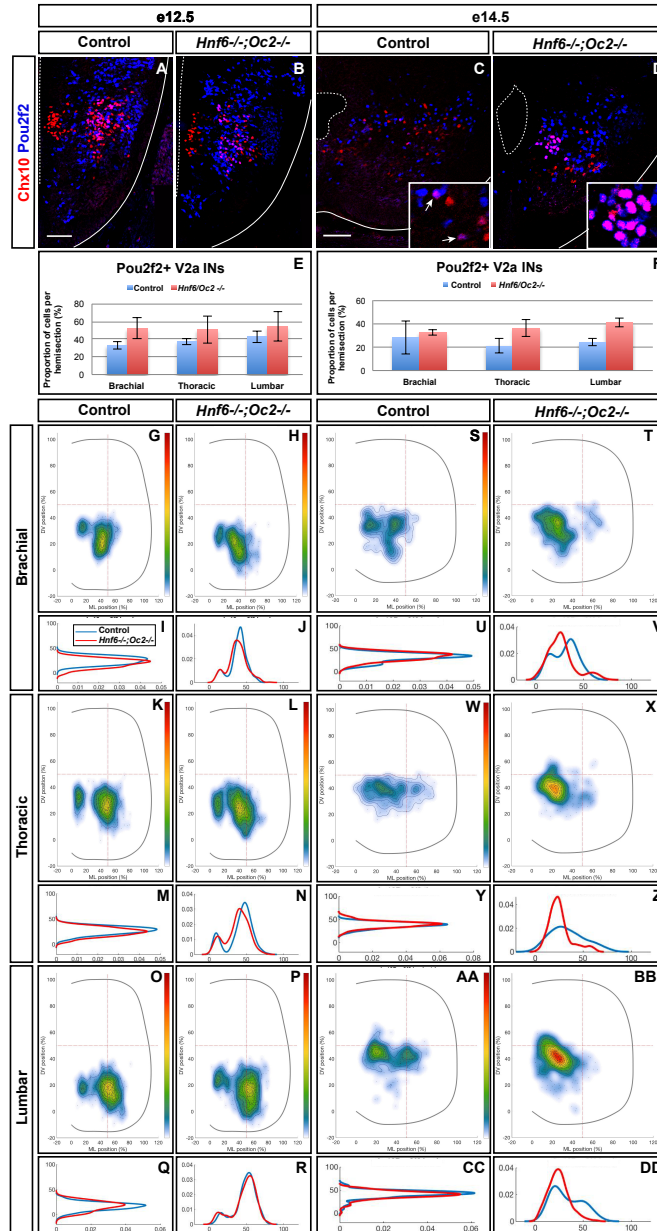
### **Pou2f2-positive V2 interneurons are mislocated in the absence of OC factors**

To assess whether increased *Pou2f2* expression in *Hnf6<sup>-/-</sup>;Oc2<sup>-/-</sup>* spinal cords corresponded to an expansion of *Pou2f2* distribution in V2 interneurons or an upregulation in its endogenous expression territory, we first quantified the number and distribution of *Pou2f2*-containing Chx10+ cells at e12.5 and e14.5 (Figure 36; Supplementary Figure S9). Immunofluorescence experiments demonstrated that *Pou2f2* is present in V2a interneurons in control embryos (Figure 36A,C; Supplementary Figure S9A,D). They also confirmed increased *Pou2f2* production in the ventral regions of the spinal cord in OC mutant embryos (Figure 36A-F). However, the increase in *Pou2f2*-positive V2a interneurons was not statistically significant (Figure 36E-F; Supplementary Figure S9). This suggested that, in the absence of OC factors, *Pou2f2* is upregulated but only modestly expanded in the V2a population. In contrast, the distribution of *Pou2f2*-positive V2a interneurons was affected (Figure 36A-D, 36G-DD). At e12.5, cells in the central clusters were slightly reduced at brachial and at thoracic levels (Figure 36G-R). In contrast, at e14.5, a majority of V2a containing *Pou2f2* settled in a medial position (Figure 36S-DD), reminiscent of the distribution defects observed for the whole V2a population (Figure 33).

Similarly, *Pou2f2* was detected in V2b interneurons in control embryos, although in a more restricted number of cells (Figure 37A-F). In the absence of OC factors, the number of *Pou2f2*-positive V2b cells was not significantly increased (Figure 37E-F) but, as observed for V2a, this subset of V2b interneurons was slightly mislocated with cells more central at e12.5 and more clustered on the medio-lateral axis at e14.5 (Figure 37G-DD). Taken together, these observations suggest that *Pou2f2* may contribute to control V2 interneuron migration during spinal

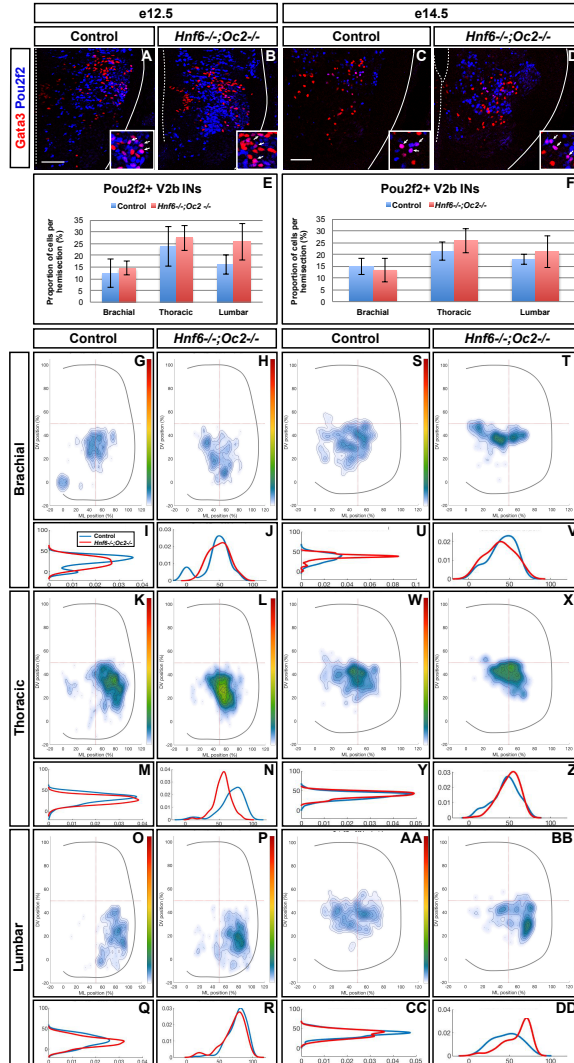
## Results

cord development and that increased *Pou2f2* expression in these cells could participate in alterations of V2 distribution in the absence of OC factors.



**Figure 36: The *Pou2f2*+ V2a interneurons are mislocated in the absence of OC factors. (A-F)** Immunolabelings and quantification of *Pou2f2*+ V2a interneurons in control or *Hnf6*<sup>-/-</sup>;*Oc2*<sup>-/-</sup> mutant embryos. At e12.5 (A-B) and e14.5 (C-D), *Pou2f2* is detected in V2a *Chx10*<sup>+</sup> interneurons, and the number of *Pou2f2*-containing *Chx10*<sup>+</sup> cells trends to increase but is not significantly different in the absence of OC factors (E-F). (G-DD) Distribution of *Pou2f2*+ V2a interneurons on transverse section of the spinal cord in control or *Hnf6*<sup>-/-</sup>;*Oc2*<sup>-/-</sup> double-mutant embryos. One-dimension graphs (lower) show density distribution on the dorso-ventral (left) or the medio-lateral (right) axis of the spinal cord. (G-R) At e12.5, cells in the central clusters are slightly reduced at brachial and at thoracic levels in the absence of OC factors (n=3, p≤0.001 at brachial and thoracic levels; p=4051 at lumbar level). (S-DD) At e14.5, a vast majority of V2a containing *Pou2f2* settle in a more medial position in *Hnf6*<sup>-/-</sup>;*Oc2*<sup>-/-</sup> spinal cords (n=3, p≤0.001). Mean values ± SEM. Scale bar = 50 μm.

## Results



**Figure 37: The Pou2f2+ V2b interneurons are mislocated in the absence of OC factors. (A-F)** Immunolabelings and quantification of Pou2f2+ V2b interneurons in control or *Hnf6*<sup>-/-</sup>;*Oc2*<sup>-/-</sup> mutant embryos. At e12.5 (A-B) and e14.5 (C-D), Pou2f2 is present in V2b Gata3+ interneurons, but the number of Pou2f2+ V2b cells was not significantly increased (E-F). (G-DD) Distribution of Pou2f2+ V2b interneurons on transverse section of the spinal cord in control or *Hnf6*<sup>-/-</sup>;*Oc2*<sup>-/-</sup> double-mutant embryos. One-dimension graphs (lower) show density distribution on the dorso-ventral (left) or the medio-lateral (right) axis of the spinal cord. (G-R) At e12.5, Pou2f2-containing V2b are more central and slightly more ventral in the absence of OC factors (n=3, p≤0.001). (S-DD) At e14.5, this subset of V2b interneurons is more clustered on the medio-lateral axis in *Hnf6*<sup>-/-</sup>;*Oc2*<sup>-/-</sup> spinal cords (n=3, p≤0.001). Mean values ± SEM. Scale bar = 50 μm.

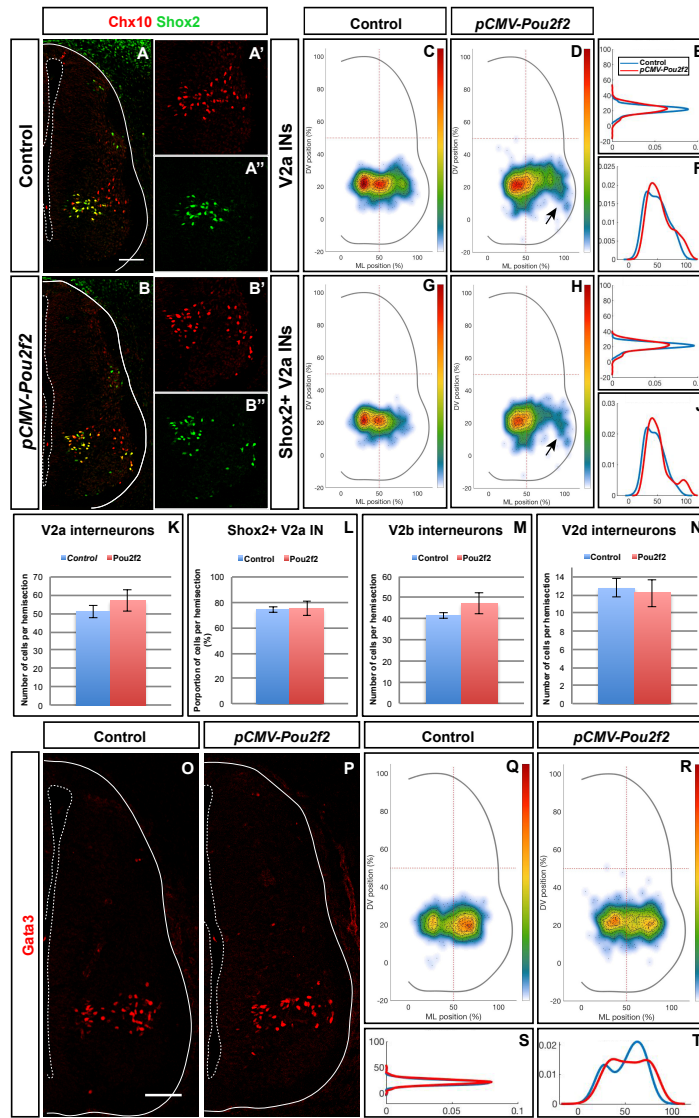


**Pou2f2 regulates the distribution of spinal V2 interneurons**

To determine whether Pou2f2 is able to modulate V2 interneuron distribution, we expressed *Pou2f2*, which is not present in the chicken genome (data not shown), in the chicken embryonic spinal cord (Supplementary Figure S10). Pou2f2 did not impact on the number of V2a, Shox2-containing V2a, V2b or V2d interneurons (Figure 38A-B", 8K-P). In contrast, it did alter V2 interneuron location. In HH27-28 control spinal cord, V2a and Shox2-containing V2a interneurons were distributed in 2 closely connected clusters on the medio-lateral axis of the neuroepithelium. In electroporated spinal cord, lateral migration was increased and a majority of V2a and Shox2-positive V2a interneurons were clustered in a single group in a central position (Figure 38A-J) with ectopic lateral extensions (arrows in Figure 38D,H). In control spinal cord, V2b were distributed in 2 groups along the medio-lateral axis with a majority of cells in the lateral cluster. In electroporated spinal cord, lateral migration of the V2b interneurons was reduced and a majority of cells were located in the medial cluster (Figure 38O-T). Thus, consistent with our observation in OC mutant embryos, increased Pou2f2 seems to modulate migration of V2 interneurons in the developing spinal cord.

To confirm the influence of Pou2f2 on V2 migration, we studied V2 distribution in mouse embryos devoid of Pou2f2 (Corcoran et al., 1993) at e12.5. Absence of Pou2f2 did not impact on the number of V2a, Shox2-positive V2a or V2d interneurons (Figure 39A-G) nor on the cMaf<sup>+</sup> or Maf<sup>+</sup> V2a subsets (Supplementary Figure S11). In contrast, V2a distribution was affected in *Pou2f2* mutants. As compared to the two V2a clusters observed in control embryos, Chx10<sup>+</sup> cells were relatively more abundant in the medial cluster in *Pou2f2* mutant embryos (Figure 39H-S). Similarly, Shox2<sup>+</sup> V2a remained more medial at brachial level, but migrated more laterally at lumbar level (Figure 39T-EE).

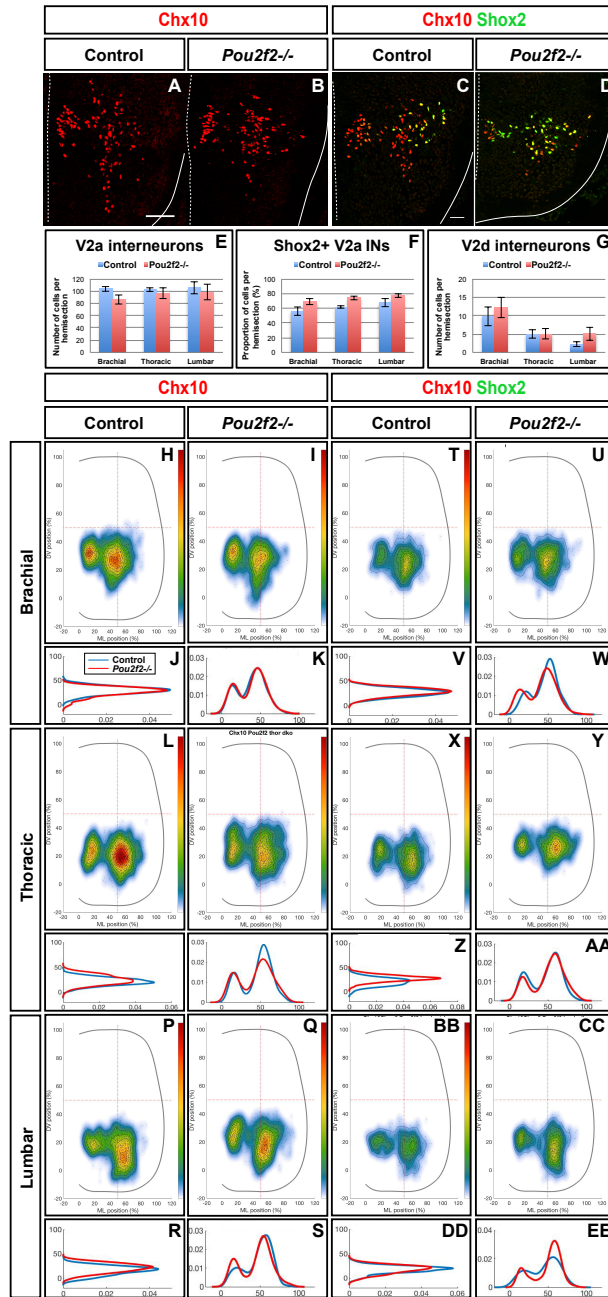
## Results



**Figure 38: V2 interneuron distribution is altered after misexpression of *Pou2f2*.** Overexpression of *Pou2f2* in chick embryonic spinal cord after electroporation at HH14-16 and immunolabelings 72 hours after electroporation. (A-B, O-P) At HH27-28, *Pou2f2* overexpression does not impact the number of V2a (Chx10+) (K), Shox2+ V2a (L), V2b (M) or V2d (N) interneurons. (C-J) In contrast, it alters V2 distribution. (C-J) In control spinal cord, V2a and Shox2+ V2a interneurons are distributed in two closely connected clusters on the medio-lateral axis of the neuroepithelium. In electroporated spinal cord, lateral migration is increased and a majority of V2a and Shox2+ V2a interneurons are clustered in a single central group with ectopic lateral extensions (arrows; n=3, p<0.001). (Q-T) In control spinal cord, V2b are distributed in two

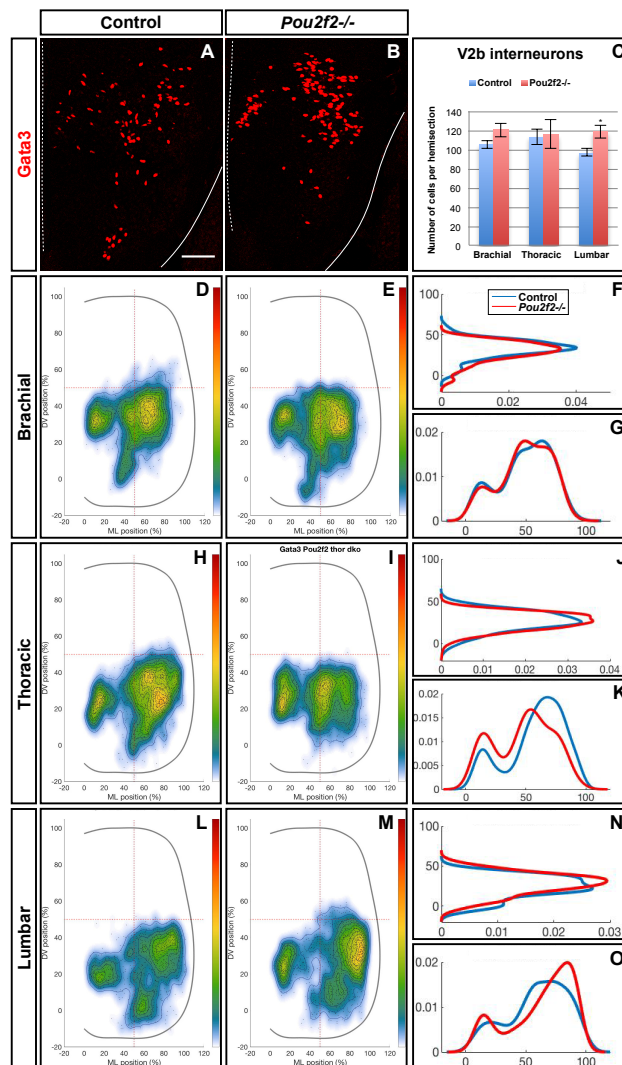
groups along the medio-lateral axis with a majority of cells in the lateral cluster. In electroporated spinal cord, lateral migration of the V2b interneurons is reduced and a majority of cells is located in the medial cluster (n=3, p<0.001). Mean values  $\pm$  SEM. Scale bar = 50  $\mu$ m.

## Results



## Results

Consistent observations were made for V2b and V2c interneurons (Figure 40). Although the number of V2b, V2c or MafA+ V2b cells was not changed in the absence of Pou2f2 (Figure 40A-C; Supplementary Figure S12), V2b cells remained more medial at thoracic levels but migrated more laterally at lumbar levels (Figure 40D-O). Taken together, these observations demonstrate that Pou2f2 regulate the distribution of V2 interneurons during spinal cord development.



**Figure 40: Pou2f2 regulate the distribution of V2b interneurons. (A-G)** Immunolabelings and quantification of V2b interneurons in control or *Pou2f2*<sup>-/-</sup> mutant embryos. At e12.5, the production of the Gata3+ V2b interneurons is not affected by the absence of Pou2f2. **(D-O)** Distribution of V2b interneurons on transverse section of the spinal cord in control or *Pou2f2*<sup>-/-</sup> mutant embryos. One-dimension graphs (lower) show density distribution on the dorso-ventral (left) or the medio-lateral (right) axis of the spinal cord. **(D-O)** The distribution of V2b cells is altered in *Pou2f2*<sup>-/-</sup> mutants, as V2b interneurons remained more medial at thoracic level but migrated more laterally at lumbar level (n=3, p<0.001). Mean values ± SEM. Scale bar = 50 μm.

### **3.2.4. Discussion**

In the recent years, several studies demonstrated that proper distribution of neuronal populations and subpopulations in the developing spinal cord is critical for adequate formation of spinal circuits (Bikoff et al., 2016; Goetz et al., 2015; Hayashi et al., 2018; Hilde et al., 2016; Surmeli et al., 2011; Tripodi et al., 2011). However, the genetic programs that control the diversification of spinal neuronal populations into specialized subpopulations and the proper settling of these neuronal subsets in the spinal parenchyma remain elusive. Here, we provide evidence that OC transcription factors regulate the diversification of spinal V2 INs, and that a genetic cascade involving OC factors and their downstream target Pou2f2 controls the distribution of V2 INs in the developing spinal cord.

#### **Control of V2 IN diversification by the OC factors**

Cardinal populations of spinal ventral INs have been well characterized, and their global contribution to the activity of motor circuits has been extensively studied (reviewed in (Boije and Kullander, 2018; Gosgnach et al., 2017; Ziskind-Conhaim and Hochman, 2017). However, more recently, the idea emerged that these cardinal populations are not homogeneous ensembles but rather contain multiple cellular subsets with distinct molecular identities and functional properties. V0 INs are constituted of two major populations, the inhibitory V0<sub>D</sub> and the excitatory V0<sub>V</sub> neurons, which control left-right alternation of motor circuit activation at low or high frequencies, respectively (Talpalar et al., 2013). V1 INs, a major inhibitory population that controls motor output, can be fractionated into ~50 distinct subsets based on the combinatorial expression of 19 transcription factors. These subsets exhibit distinct electrophysiological properties and highly structured

spatial distribution that constraints somato-sensory afferences (Bikoff et al., 2016). Furthermore, they are differentially distributed along the anteroposterior axis of the spinal cord (Francius et al., 2013; Sweeney et al., 2018), suggesting differential contribution to the motor output at thoracic or at limb levels of the spinal cord (Sweeney et al., 2018). Similarly, V2a INs comprise two major divisions, namely type I and type II V2a cells, that are arrayed in counter-gradients along the antero-posterior axis of the spinal cord and activate different patterns of motor output at brachial and lumbar levels. Furthermore, these two large divisions can themselves be fractionated at birth into 11 subsets characterized by distinct combinations of markers, differential segmental localization and specific distribution patterns on the medio-lateral axis of the spinal cord (Hayashi et al., 2018). In the zebrafish, 3 distinct subclasses of V2a INs participate in separate microcircuit modules driving slow, intermediate or fast motor neuron activity (Ampatzis et al., 2014). Finally, V3 INs segregate into physiologically and topographically distinct clusters along the dorso-ventral axis of the spinal cord, each showing distinctive maturation processes and likely playing different roles in motor activity (Borowska et al., 2015; Borowska et al., 2013). Taken together, these observations suggest that cardinal IN populations only constitute the first organization level of functionally distinct neuronal subsets that contribute to diversity and flexibility within spinal motor circuits. We show here that, as observed in spinal motor neurons and other IN populations (Francius and Clotman, 2010; Francius et al., 2013; Kabayiza et al., 2017), OC factors are also detected in subsets of V2 INs. As reported for the V1 population (Bikoff et al., 2016; Sweeney et al., 2018), OC proteins may define functionally-relevant V2 subpopulations, although possible correlation with the recently-identified V2a subsets (Hayashi et al., 2018) remains to be investigated.

However, for each of these cardinal populations, the genetic programs that contribute to their diversification into multiple subpopulations remain unknown. Our observations demonstrate that OC factors contribute to the diversification of V2 INs. Normal numbers of cardinal V2a and V2b cells were generated in OC mutant embryos, suggesting that these factors do not contribute to the production of V2 cells (Clovis et al., 2016; Lee et al., 2008; Thaler et al., 2002) nor to the segregation of the V2a and V2b lineages through differential activation of Notch signaling (Del Barrio et al., 2007; Joshi et al., 2009; Misra et al., 2014; Peng et al., 2007). In contrast, V2a subpopulations characterized by the presence of MafA or cMaf were strongly depleted in the absence of OC proteins. Uncomplete knowledge of the whole collection of V2a subsets prevented to assess whether this reduction in specific subpopulations were compensated for by an expansion of neighboring subsets. Furthermore, MafA and cMaf were not included among the markers that fractionate at birth the V2a population into 11 distinct subgroups (Hayashi et al., 2018) and the matching between these subpopulations therefore remains to be investigated. Nevertheless, these observations suggest that, as previously observed for spinal motor neurons (Roy et al., 2012) and dorsal INs (Kabayiza et al., 2017), OC factors contribute to the diversification of V2a INs during development. In addition, the production of V2c cells was delayed in OC mutant embryos, although V2b that are supposed to constitute the source of V2c (Panayi et al., 2010) were timely generated. This points to a specific contribution of OC protein to the development of V2c INs, the mechanism of which is currently unknown.

**Control of V2 IN migration by the OC factors**

Beside diversification, 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. Extensor and flexor premotor INs segregate along the medio-lateral axis of the spinal cord (Tripodi et al., 2011). Premotor INs that convey information to motor neurons innervating axial muscles reside in symmetrically balanced locations while limb-innervating motor neurons are mainly innervated by ipsilateral premotor INs (Goetz et al., 2015). The settling position of distinct V1 IN subsets is predictive of the status of motor and sensory neuron input (Bikoff et al., 2016). Among V2 INs, type I cells are distributed as an increasing rostro-caudal gradient and contribute to local motor circuits, whereas type II cells are arrayed as an increasing caudo-rostral gradient and project both to motor neurons and supraspinally (Hayashi et al., 2018), likely sending motor efference copies to the cerebellum via the lateral reticular nucleus (Azim et al., 2014; Pivetta et al., 2014). Furthermore, ventral and dorsal V3 INs are differentially activated during running and swimming, suggesting specific contribution of spatially distinct V3 subsets to different motor behavior (Borowska et al., 2013). Finally, inhibitory sensory relay neurons of lamina V characterized by the presence of SATB2 are abnormally located upon *SATB2* inactivation and this, along with changes in molecular identity, perturbs pre- and post-synaptic connectivity (Hilde et al., 2016). Taken together, these recent data support a model wherein correct localization of spinal IN subsets is critical for proper formation of sensory and sensory-motor circuits, reminiscent of the requirement of correct motor neuron positioning for the establishment of adequate sensory inputs (Dasen, 2017; Surmeli et al., 2011). This highlights 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. *Sim1* regulate ventro-dorsal migration of the V3 IN subsets (Blacklaws et al., 2015). Similarly, *SATB2* control the position of inhibitory sensory relay INs along the medio-lateral axis of the spinal cord (Hilde et al., 2016). Here, we provide evidence that the OC factors control a genetic program that regulates proper positioning of V2 INs during embryonic development. In the absence of OC proteins, a fraction of V2a INs remained in a more medial location, expanding the medial cluster containing locally-projecting cells at the expense of the lateral cluster that comprises the supraspinal-projecting V2a INs (Hayashi et al., 2018). V2b alterations were less spectacular, although ventral and dorsal contingents were reduced and the cell distribution in the central cluster was altered. Variability in the alterations observed at e12.5 and e14.5 suggests that, depending on the population considered, migration of earlier- or later-migrating neurons may be differently affected by the absence of OC proteins. Functional distinctions between V2b subsets have not been reported to date. However, given our current knowledge regarding the diversification and the impact of localization of V1 and V2a IN subpopulations on proper circuit formation (Bikoff et al., 2016; Hayashi et al., 2018), we hypothesize that even slight perturbations in the distribution of small IN subsets are likely to result in alterations in spinal microcircuit development. Our observations are consistent with the contribution of OC factors to the migration of several populations of spinal dorsal INs (Kabayiza et al., 2017). This raises the question whether similar cues might be regulated by identical genetic programs and used by different IN populations to organize proper distribution of ventral and dorsal IN subsets in the developing spinal cord. Identification of the factors downstream of OC protein involved in the control of neuronal migration will be necessary to answer this question.

**A OC-Pou2f2 genetic cascade regulates the migration of V2 INs**

Therefore, we attempted to identify genes downstream of OC factors and possibly involved in the control of IN migration using a global approach comparing the transcriptome of whole spinal cords isolated from control or OC-deficient embryos. However, we were unable to detect significant changes in the expression of known regulators of neuronal migration. This suggests that different actors may be active downstream of OC proteins in distinct IN subsets to regulate proper neuronal distribution. In contrast, we uncovered that expression of the transcriptional regulator *Pou2f2* is repressed by OC factors in multiple IN populations. Surprisingly, our data demonstrated that variant *Pou2f2* isoforms are produced in the developing spinal cord as compared to B lymphocytes (Hatzopoulos et al., 1990; Lillycrop and Latchman, 1992; Liu et al., 1995; Stoykova et al., 1992; Wirth et al., 1991), and that these spinal variants are regulated by OC proteins. Spinal-enriched transcripts encode *Pou2f2* proteins containing additional peptidic sequences upstream of the POU-specific domain and of the homeodomain. Furthermore, exon 1 is different and corresponds to sequences located ~47kb upstream of the transcription initiation site used in B cells (data not shown) in the mouse genome, suggesting that OC regulate *Pou2f2* expression from an alternative promoter. However, we cannot exclude that additional exon(s) could be present upstream of the identified sequences, and determination of the regulating sequences targeted by the OC protein will require thorough characterization of the produced transcripts. In addition, we cannot rule out indirect regulation of *Pou2f2* expression by the OC factors, as these proteins are usually considered to be transcriptional activators rather than repressors (Beaudry et al., 2006; Jacquemin et al., 2000; Jacquemin et al., 2003a; Lannoy et al., 2000; Pierreux et al., 2004; Roy et al., 2012). Whether divergent N-terminal sequence and additional peptidic sequence upstream of the POU-specific domain

modifies the activity or the binding specificity of the Pou2f2 proteins also awaits further investigations.

Nevertheless, our observations demonstrate that Pou2f2 is downstream of OC factors in the V2 INs and also contributes to regulate the distribution of V2 INs during embryonic development. The number of Pou2f2-containing V2 was not significantly increased in OC mutant spinal cords, suggesting that the absence of OC protein resulted in derepression of Pou2f2 production in its endogenous expression domain rather than ectopic activation in other V2 subsets. Increased production of Pou2f2 in the chicken embryonic spinal cord resulted in alterations in the localization of V2 populations without any change in cell number, pointing to a possible contribution of Pou2f2 to the regulation of V2 migration downstream of OC factors. Consistently, V2 distribution was perturbed in *Pou2f2* mutant embryos without any alteration in V2 population or subpopulation cell numbers, demonstrating the involvement of Pou2f2 in the control of V2 IN distribution. Hence, we uncovered a genetic cascade comprising OC and Pou2f2 transcription factors that ensures proper migration of V2 cells during spinal cord development. This program may not be restricted to V2 cells, as diversification and distribution of dorsal INs and of motor neurons are also altered in the absence of OC factors (Kabayiza et al., 2017; Roy et al., 2012) and as *Pou2f2* expression in the OC mutant spinal cord is increased in multiple neuronal populations (Figure 35D-I). Therefore, evaluating the consequences of V2 IN defects on motor circuit activity in *OC* or *Pou2f2* mutants would necessitate conditional inactivation of these transcription factors in this specific population. Whether V2 localization defects are associated with axon guidance perturbations, as observed for V3 INs in the absence of Sim1 (Blacklaws et al., 2015), will require genetic labeling of V2 axonal projections.

Localization defects of specific neuronal populations have previously been reported in the absence of OC factors. Dopaminergic neurons of the A13 nucleus migrate aberrantly in OC mutant embryos, resulting in the disaggregation of the nucleus and loss of dopaminergic cell phenotype (Espana and Clotman, 2012b). Similarly, neurons of the Locus Coeruleus, the largest noradrenergic nucleus of the CNS, migrate caudally beyond their normal position in the rostral hindbrain of OC deficient individuals, resulting in the loss of their catecholaminergic characteristics (Espana and Clotman, 2012a). Final positioning of the Purkinje cells during postnatal maturation of the cerebellum was defective in the absence of HNF-6 (Audouard et al., 2013). Finally, distribution defects of spinal dorsal INs in OC mutant embryos have recently been reported (Kabayiza et al., 2017). Hence, OC factors seem critical for proper localization of multiple neuronal populations throughout the CNS. Whether identical migration regulators are controlled by OC proteins in different neuronal populations remains to be determined. Comparison of downstream targets of OC and of Pou2f2 that would be oppositely regulated in the respective mutants may constitute a strategy to identify these regulators.

### **3.2.5. Materials and methods**

#### **Ethics statement and mouse lines**

All experiments were strictly performed in accordance with the European Community Council directive of 24 November 1986 (86-609/ECC) and the decree of 20 October 1987 (87-848/EEC). Mice were raised in our animal facilities and treated according to the principles of laboratory animal care, and experiments and mouse housing were approved by the Animal Welfare Committee of Université catholique de Louvain (Permit Number: 2013/UCL/MD/11 and 2017/UCL/MD/008). The day of vaginal plug was considered to be embryonic day (e) 0.5. A minimum of three embryos of the same genotype was analyzed in each experiment. The embryos were collected at e12.5 and e14.5. The *Hnf6;Oc2* and the *Pou2f2* mutant mice were previously described (Clotman et al., 2005; Corcoran et al., 1993; Jacquemin et al., 2000). The mice and the embryos were genotyped by PCR (primer information available on request).

#### ***In situ* hybridization (ISH) and immunofluorescence labelings**

For ISH, the 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, embedded and frozen in PBS/15% sucrose/7.5% gelatin. Fourteen µm section were prepared and ISH was performed as previously described (Beguín et al., 2013; Francius et al., 2016; Pelosi et al., 2014) with DIG-conjugated *Pou2f2* (NM\_011138.1, nucleotides 604-1187) or *Pou2f2* exon 5b (XM\_006539651.3, nucleotides 643-876) antisense RNA probes.

For immunofluorescence, collected embryos were fixed in 4% PFA/PBS for 25 or 35 minutes according to their embryonic stage and processed as for ISH.

Immunolabeling was performed on 14  $\mu\text{m}$  serial cryosections as previously described (Francius and Clotman, 2010). Primary antibodies against the following proteins were used: Chx10 (sheep; 1:500; Exalpha Biologicals #X1179P), Foxp1 (goat; 1:1000; R&D Systems #AF4534), Gata3 (rat; 1:50; Absea Biotechnology #111214D02), GFP (chick; 1:1000; Aves Lab #GFP-1020), HNF6 (guinea pig; 1:2000; (Espana and Clotman, 2012b); or rabbit; 1:100; Santa Cruz #sc-13050; or sheep; 1:1000 R&D Systems #AF6277), cMaf (rabbit; 1:3000; kindly provided by H. Wende), MafA (guinea pig; 1:500; kindly provide by T. Müller), OC2 (rat; 1:400; (Clotman et al., 2005); or sheep; 1:500; R&D Systems #AF6294), OC3 (guinea pig; 1:6000; (Pierreux et al., 2004)), Pou2f2 (rabbit; 1:2000; Abcam #ab178679), Shox2 (mouse; 1:500; Abcam #ab55740), Sox1 (goat; 1:500; Santa Cruz #sc-17318). Secondary antibodies donkey anti-guinea pig/AlexaFluor 488, 594 or 647, anti-mouse/AlexaFluor 488, 594 or 647, anti-rabbit/AlexaFluor 594 or 647, anti-goat/AlexaFluor 488, anti-rat/AlexaFluor 647, anti-sheep/AlexaFluor 594 or 647, and goat anti-mouse IgG2A specific/AlexiaFluor 488, purchased from ThermoFisher Scientific or Jackson Laboratories were used at dilution 1:2000 or 1:1000, respectively.

### ***In ovo* electroporation**

*In ovo* electroporations were performed at stage HH14-16, as previously described (Roy et al., 2012). The coding sequence of the S\_Pou2f2.4 transcript was amplified by overlapping-PCR using: forward 5' GCTCTGTCTGCCCCAAGAGAAA 3' and reverse 5' GTTGGGACAAGGTGAGCTGT 3' primers for the 5' sequence, forward 5' CCACCATCACAGCCTACCAG 3' and reverse 5' ATTATCTCGAGCCAGCCTCCTTACCCTCTCT 3' (designed to enable integration at the *Xho*I restriction site of the pCMV-MCS vector) primers for the 3' sequence. This sequence was first subcloned in a pCR® II-Topo® vector (Life Technologies, 45-0640)

for sequencing then subcloned at the *EcoRI* (from the pCR<sup>®</sup> II-Topo<sup>®</sup> vector) and *XhoI* restriction sites of a pCMV-MCS vector for the *in ovo* electroporation. The pCMV-Pou2f2 (0.5 mg/ml) vector was co-electroporated with a pCMV-eGFP plasmid (0.25mg/ml) to visualize electroporated cells. The embryos were collected 72 hours (HH27-28) after electroporation, fixed in PBS/4%PFA for 45 minutes and processed for immunofluorescence labelings as previously described (Francius and Clotman, 2010).

### Imaging and quantitative analyses

Immunofluorescence and ISH images of cryosections were acquired on an EVOS FL Auto Imaging System (Thermo Fisher Scientific) or a Confocal laser Scanning biological microscope FV1000 Fluoview with the FV10-ASW 01.02 software (Olympus). The images were processed with Adobe Photoshop CS5 software to match brightness and contrast with the observation. Quantifications were performed on red or green or blue layer of acquired confocal images and double or triple labeled cells were processed by subtractive method (Francius and Clotman, 2010). For each embryo ( $n \geq 3$ ), one side of three to five sections at brachial, thoracic or lumbar level were quantified using the count analysis tool of Adobe Photoshop CS5 software. Raw data were exported from Adobe Photoshop CS5 software to Sigma Plotv12.3 software to perform statistical analyses. The histograms were drawn with Microsoft Excel. Adequate statistical tests were applied based on the number of comparisons and on the variance in each group. For analysis of cell quantifications based on comparison of two groups (control or mutant), standard Student's *t*-tests or Mann-Whitney U tests were performed. Quantitative analyses were considered significant at  $p \leq 0.05$  (Three asterisks (\*\*\*) indicate  $p \leq 0.001$ ).

Quantitative analyzes of interneuron spatial distribution were performed as previously described (Kabayiza et al., 2017). Statistical analyses of ventral IN distribution were performed using a two-sample Hotelling's T<sup>2</sup>, which is a two-dimensional generalization of the Student's t test. The analysis was implemented using the NCSS software package.

### **Microarray analyses**

RNA was extracted from control or *Hnf6/Oc2* double-mutant spinal cords. The tissue was manually dissociated in Tripure isolation reagent (Roche, 11 667 165 001). After dissociation, chloroform (Merck Millipore, 1 02445 1000) was added to the sample, incubated at room temperature for 10 minutes and centrifugated for 10 minutes at 4°C. The aqueous phase was collected and the RNA was precipitated with isopropanol (VWR, 20880.310) and centrifugated for 15 minutes at 4°C. The pellet was washed in ethanol (Biosolve, 06250502) and centrifugated for 10 minutes at 4°C. The dried pellet was resuspended in RNase free water. The integrity of the RNA was assessed using an Agilent RNA 6000 Nano assay procedure. For microarray analyzes, the RNA was converted in single-strand cDNA, labeled using the GeneChip® WT PLUS Reagent Kit (Affymetrix) and hybridized on the GeneChip® MoGene 2.0 ST array (Affymetrix, 90 2118) using Affymetrix devices: Genechip® Fluidics Station 450, Genechip® Hybridization oven 640, Affymetrix Genechip® scanner and the Expression Console software. The analyzes were performed using the R software. Microarray data have been deposited in the GEO repository (accession number: GSE117871).

### **Amplification of *Pou2f2* isoforms and sequencing**

Fragments of the different *Pou2f2* isoforms were amplified by RT-PCR from RNA of control B lymphocytes or embryonic spinal cords purified as described above.



cDNA was obtained from 500 ng of RNA using the iScript™ Reverse transcriptase and the 5x iScript™ reaction mix (BioRad). Conserved or divergent sequences of *Pou2f2* isoforms were amplified as shown in Table 7 using a GoTaq® Green master mix (Promega, M712) or a Q5® Hot Start High-Fidelity DNA Polymerase (New England BioLabs® Inc, M0493S) (primer information available on request). Sequencing of the spinal *Pou2f2* exons was outsourced to Genewiz.

For quantitative RT-PCR, RNA was extracted from control (n = 5) or Hnf6/Oc2 double-mutant (n = 4) spinal cords and purified and retrotranscribed as described above. Quantitative real-time PCR was performed on 1/100 of the retrotranscription reaction using iTaq™ universal SYBR® Green Supermix (BioRad, 172-5124) on a CFX Connect™ Real-Time System (BioRad) with the BioRad CFX Manage 3.1 software. Each reaction was performed in duplicate and relative mRNA quantities were normalized to the housekeeping gene RPL32. The B cell *Pou2f2* isoforms were amplified using the following primers: forward 5' TGGTTCATTCCAGCATGGGG 3', reverse 5' TCCAGACTTTGCTTCTCGGC 3' and the spinal *Pou2f2* isoforms using: forward 5' CCACCATCACAGCCTACCAG 3', reverse 5' GAGCTGGAGGAGTTGCTGTA 3' and RPL32 using: forward 5' GGCACCAGTCAGACCGATAT 3', reverse 5' CAGGATCTGGCCCTTGAAC 3'. Relative expression changes between conditions were calculated using the DDCT method. All changes are shown as fold changes.

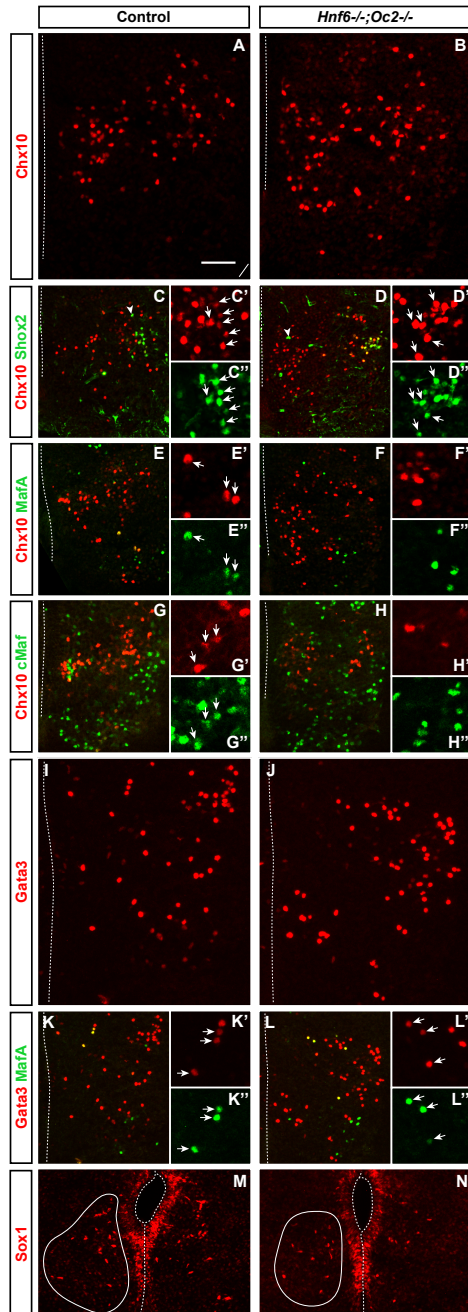
### **3.2.6. Acknowledgements**

We thank members of the NEDI lab for material, technical support and discussions. We are grateful to Drs. T. Müller and H. Wende for kindly providing the guinea pig anti MafA and the rabbit anti cMaf antibodies, respectively, to Drs. F. Lemaigre and P. Jacquemin for the Hnf6/Oc2 double mutant mice, to Dr. C. Pierreux for the pCMV-MCS, to Dr. J. Ambroise for assistance with the microarray analyses and to Dr. L. Dumoutier for cDNAs of B lymphocytes.

Work in the F.C. laboratory was supported by grants from the "Fonds spéciaux de recherche" (FSR) of the Université catholique de 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, Belgium), by the "Actions de Recherche Concertées (ARC)" #10/15-026 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'" and by the Association Belge contre les Maladies neuro-Musculaires. L.M.C. acknowledges funding from the Australian Government (NHMRC IRIIS and research grants #637306 and #575500) and Victorian State Government Operational Infrastructure Support. S.D. and C.B. hold PhD grants from the Fonds pour la Recherche dans l'Industrie et l'Agriculture (F.R.S.-FNRS, Belgium), M.H.-F. was a Postdoctoral Researcher of the F.R.S.-FNRS, F.C. is a Senior Research Associate of the F.R.S.-FNRS.

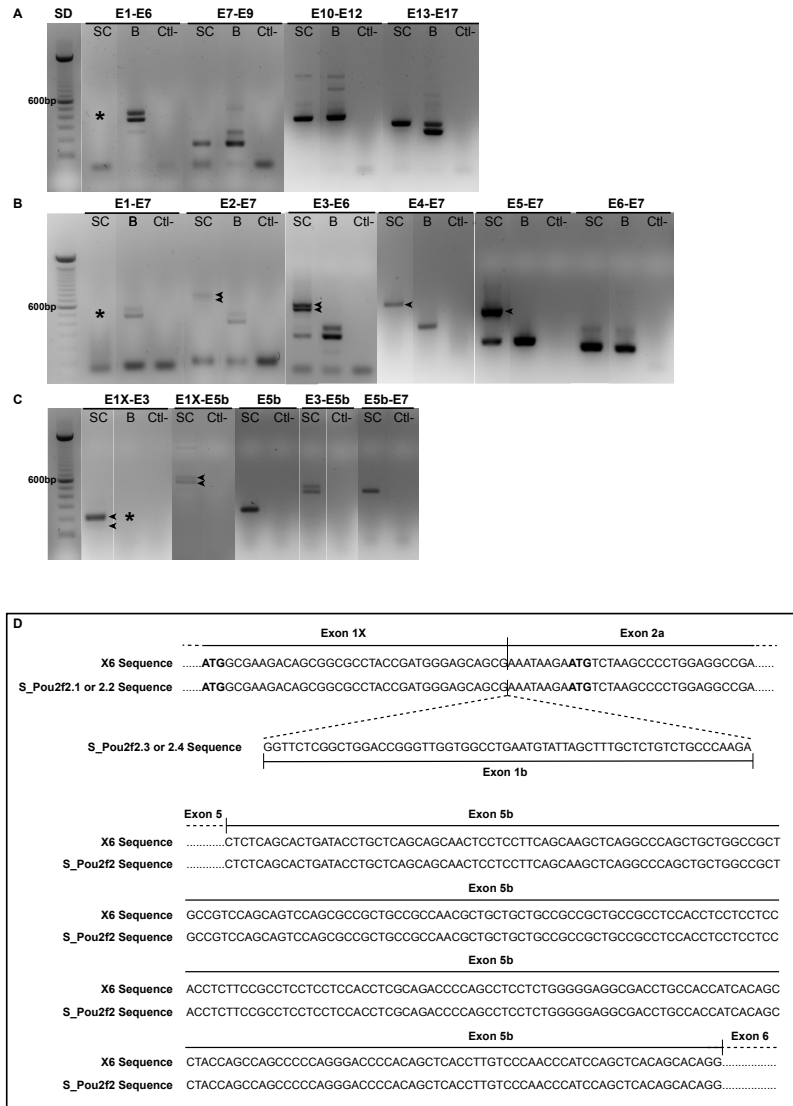
**Competing interests:** the authors declare no competing interests

## 3.2.7. Supplementary Figures



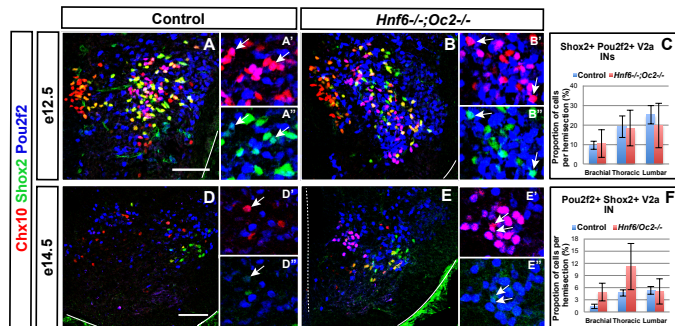
**Supplementary Figure S7: OC factors regulate the diversification of the V2 interneurons. (A-H'')** Immunolabelings of the V2a generic marker Chx10 and markers of V2a subpopulations on transverse spinal cord sections (brachial or thoracic levels) control or *Hnf6*<sup>-/-</sup>;*Oc2*<sup>-/-</sup> mutant embryos at e14.5. Quantifications are shown in Fig 1. V2a (A-B), Shox2+ V2a (arrows) and V2d interneurons (C- D'', arrowheads) are present in both control and double mutant embryos. (E-E'') MafA is present in a V2a subpopulation in control embryos (arrows), (F-F'') but is not detected in Chx10+ cells in the absence of OC factors. (G-G'') Similarly, cMaf is present in a subpopulation of V2a interneurons in control embryos (arrows), (H-H'') which is not the case in *Hnf6*<sup>-/-</sup>;*Oc2*<sup>-/-</sup> embryos. (I-L'') Immunolabelings of the V2b generic marker Gata3 and the marker of V2b subpopulation, MafA. V2b (I-J'') and MafA+ V2b (K-L'', arrows) interneurons are present in control and in *Hnf6*<sup>-/-</sup>;*Oc2*<sup>-/-</sup> embryos. (M-N) The number of V2c interneurons is unchanged is similarly detected in *Hnf6*<sup>-/-</sup>;*Oc2*<sup>-/-</sup> embryos as compared to control embryos (delineated cells). Sox1 in the ventricular zone labels neural progenitors. Scale bar = 50 μm.

## Results



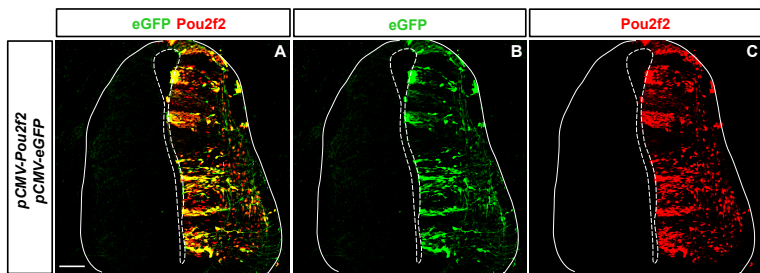
**Supplementary Figure S8: Pou2f2 RT-PCR experiments and sequence of the spinal Pou2f2 exon 1b and exon 5b.** Composite assembly of electrophoresis images of RT-PCR amplification products for *Pou2f2* isoform sequences on embryonic spinal cord or B-cell RNA samples. Water was used as a negative control (Ctl-). **(A)** Amplifications from exon 1 (E1) to E6 on embryonic spinal cord RNA samples (asterisk) fail to amplify the RNA isoforms detected in B cell samples. In contrast, amplifications from E7 to E9, from E10 to E12 and from E13 to E17 show at least one similar amplicon in spinal cord samples and in B cell samples. **(B)** Amplification from E1 (other forward primer) to E7 also fails to amplify *Pou2f2* spinal cord isoforms. In contrast, amplifications from E2, 3, 4 and 5 to E7 produce systematically longer amplicons in spinal cord samples (arrowheads) as compared to B cell samples. The E6 to E7 amplification is similar in both samples. →

## Results



**Supplementary Figure S9:** The number of Pou2f2+Shox2+ V2a interneurons is normal in *Hnf6*<sup>-/-</sup>; *Oc2*<sup>-/-</sup> mutant embryos. Immunolabelings on transverse spinal cord sections (brachial or thoracic levels) of control or *Hnf6*<sup>-/-</sup>; *Oc2*<sup>-/-</sup> mutant embryos. At e12.5 (A-C) and at e14.5 (D-F), the number of V2a

containing Shox2 and Pou2f2 is unchanged in the absence of OC factors. Mean values  $\pm$  SEM. Scale bar = 50  $\mu$ m.

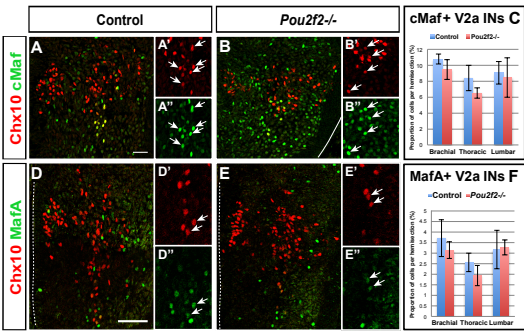


**Supplementary Figure S10.** Efficacy of pCMV-eGFP and pCMV-Pou2f2 co-electroporation in the chicken embryonic spinal cord. (A-C) eGFP (green, B) and Pou2f2 (red, C) are

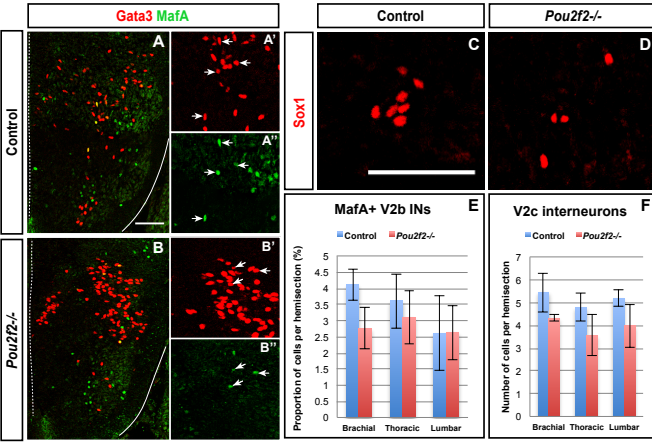
present in a vast majority of cells along the dorso-ventral axis of the spinal cord. Scale bar = 50  $\mu$ m.

(C) Amplifications from E1X (present in the X6 sequence) to E3 do not produce amplicon in B-cell samples (asterisk). Amplifications from 1X to E3 or to E5b on spinal cord samples systematically produce 2 amplicons (arrowheads). Amplifications of the E5b exon sequence, from E3 to E5b and from E5b to E7 produce expected amplicons in spinal cord samples. (D) Comparison of exon 1b and exon 5b sequences in the predicted X6 sequence and the sequenced embryonic spinal cord *Pou2f2* isoforms. Sequences of E1X, E2a and E5b exons align (100% identity) with the predicted X6 sequence. Sequence of the additional alternative exon 1b is shown. SD = Size standard, E = Exon, SC = Embryonic spinal cord, B = B lymphocytes.

Results



**Supplementary Figure S11.** The number of MafA+ or cMaf+ V2a interneurons is normal in *Pou2f2*<sup>-/-</sup> mutant spinal cords. Immunolabelings on transverse spinal cord sections (brachial or thoracic levels) of control or *Pou2f2*<sup>-/-</sup> mutant embryos at e12.5. (A-F) Absence of Pou2f2 does not impact on the number of MafA+ (A-C) or cMaf+ V2a interneurons (D-F). Mean values ± SEM. Scale bar = 50 µm.



**Supplementary Figure S12:** The number of MafA-positive V2b interneurons or of the V2c interneurons is normal in *Pou2f2*<sup>-/-</sup> mutant spinal cords. Immunolabelings on transverse spinal cord sections (brachial or thoracic levels) of control or *Pou2f2*<sup>-/-</sup> mutant embryos at e12.5. (A-B, E) The number of MafA+ V2b interneurons is not significantly altered in the absence of Pou2f2. (C-D, F) Similarly, the production of

V2c interneurons is not affected in *Pou2f2* mutants. Mean values ± SEM. Scale bar = 50 µm.

### **3.3. Onecut factors control the expression of *Nkx6.2*, which contributes to the control of the distribution of V2 interneurons in the developing spinal cord**

#### **3.3.1. Foreword**

This study is currently not intended to be submit as a publication, but could be associated to other results to be published in the future. In this work, I carried out all the experiments.

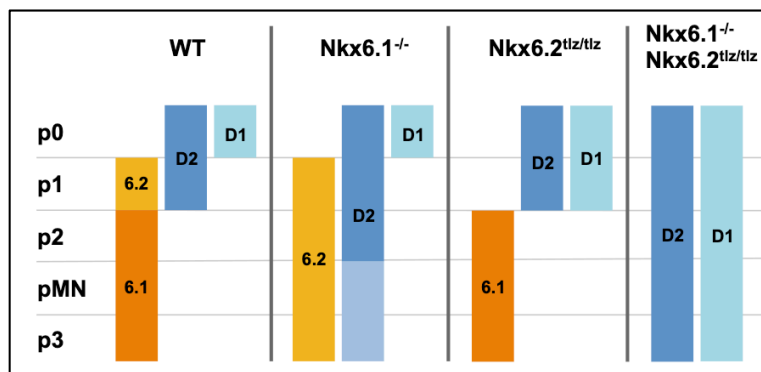
#### **3.3.2. Introduction**

The microarray result analysis allowed us to uncover another potential regulator of V2 differentiation and/or migration, *Nkx6.2*. Although *Nkx6.2* was not significantly downregulated (0.7-fold decrease), its role during ventral neural patterning and cell fate specification led us to continue the analyses of this candidate. Indeed, *Nkx6.2*, expressed in the p1 progenitor domain, controls the V1 cell fate specification and the MN production in combination with *Nkx6.1*. It is also expressed in non-characterized ventral postmitotic INs and in a motor pool of the LMCI.

*Nkx6.2* is a homeobox transcription factor expressed in progenitor and in differentiating cells in the spinal cord and the hindbrain (Cai et al., 1999; Pattyn et al., 2003; Qiu et al., 1998). As extensively demonstrated, class I and II HD transcription factors regulated by Shh are regionally expressed in the ventricular zone of the spinal cord and cross-repress each other, resulting in well-defined progenitor domain boundaries. Hence in the mouse, *Nkx6.2* expressed in the p1 progenitor domain promotes the V1 cell fate specification by repressing *Dbx1* expression and thereby preventing V0 IN production (Sander et al., 2000; Vallstedt et al., 2001). Indeed, in *Nkx6.2* mutant mice, V0 INs are produced from the p1 progenitor domain at the expense of V1 INs. In return, *Nkx6.2* is repressed

## Results

dorsally by *Dbx1* and ventrally by *Nkx6.1*, its paralog. It has been demonstrated that *Nkx6.2* plays a redundant role with *Nkx6.1* during the MN specification. Indeed, in *Nkx6.1* mutant mice, MNs are completely lost at brachial level of the spinal cord, while being partially lost at lumbar level. Moreover, *Nkx6.2* expression extends ventrally to the p3 progenitor domain in these mutants. In the *Nkx6.1*<sup>-/-</sup>;*Nkx6.2*<sup>tlz/tlz</sup> compound mutant embryos, MNs are completely lost at all levels, indicating that *Nkx6.2* partially compensates for the loss of *Nkx6.1* in MN production. The expansion of the V1 IN production in these double mutants is less important than in the simple *Nkx6.1* mutant due to the derepression of *Dbx1* by *Nkx6.2* resulting in a larger V0 IN production (Figure 41) (Vallstedt et al., 2001).



**Figure 41: Dorso-ventral patterning in wild-type or *Nkx6* mutant mouse embryos (Vallstedt et al., 2001).** In wild-type (WT) embryos, the p0 progenitor domain expresses *Dbx1* (D1) and *Dbx2* (D2), the p1 domain expresses

*Dbx2* and *Nkx6.2* (6.2), *Nkx6.1* (6.1) is expressed in p2 to p3 progenitor domains. In *Nkx6.1*<sup>-/-</sup> mutant embryos, *Dbx2* and *Nkx6.2* extend ventrally to the p2 and the p3 domains, respectively, leading to the production of V1 INs. In *Nkx6.2*<sup>tlz/tlz</sup> mutant embryos, *Dbx1* extend ventrally to the p1 domain promoting the production of V0 INs. Finally in the *Nkx6.1*<sup>-/-</sup>;*Nkx6.2*<sup>tlz/tlz</sup> compound mutant embryos, both *Dbx1* and *Dbx2* extend ventrally to the p3 progenitor domain, resulting in the production of V0 and V1 INs.

*Nkx6.2* is also expressed in a motor pool, distinct from the *Nkx6.1*+ motor pool in the LMCI, and in a ventral IN population (Cai et al., 1999; De Marco Garcia and Jessell, 2008). Nevertheless, the identity of the ventral IN population and the role of *Nkx6.2* during its development have not been yet investigated.

Although the *Nkx6.2*-null mutant mice reproduce normally and grow up

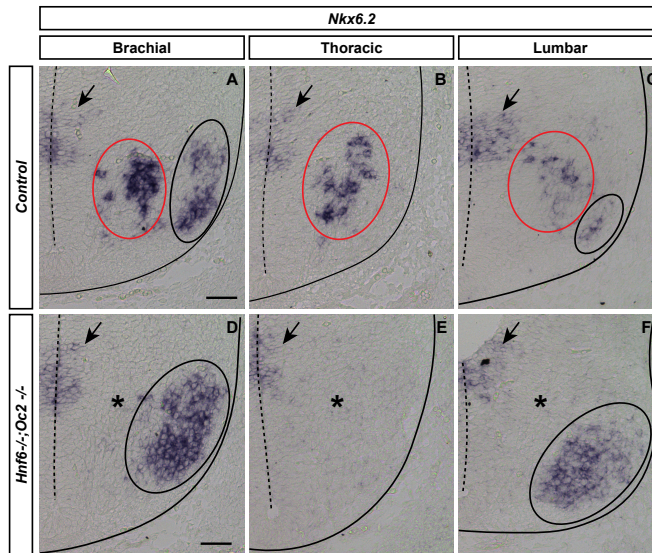


like their wild-type littermates, those mutants show coordination defects 3 weeks after birth when tested by rotarod and auditory brainstem response. These behavioral disorders were associated with myelination defects in the absence of *Nkx6.2*. Indeed, *Nkx6.2* mutant mice exhibit morphological abnormalities of the central nervous system paranodes and ectopic axoglial junctions in juxtaparanodal myelin. *Nkx6.2* regulates the expression of a paranodal microtubule destabilizing protein, *Stathmin 1*, and paranodal cell adhesion molecules, *NF155* and *contactin*, in oligodendrocytes postnatally during myelinogenesis. The expression defects of these 3 proteins could be at the origin of the paranode morphological alterations seen in *Nkx6.2*-deficient mice (Southwood et al., 2004). Furthermore, in human, a mutation in *Nkx6.2* has been discovered resulting in a truncated protein causing progressive ataxia and hypomyelination (Chelban et al., 2017).

### **3.3.3. Results**

#### **OC factors control the expression of *Nkx6.2***

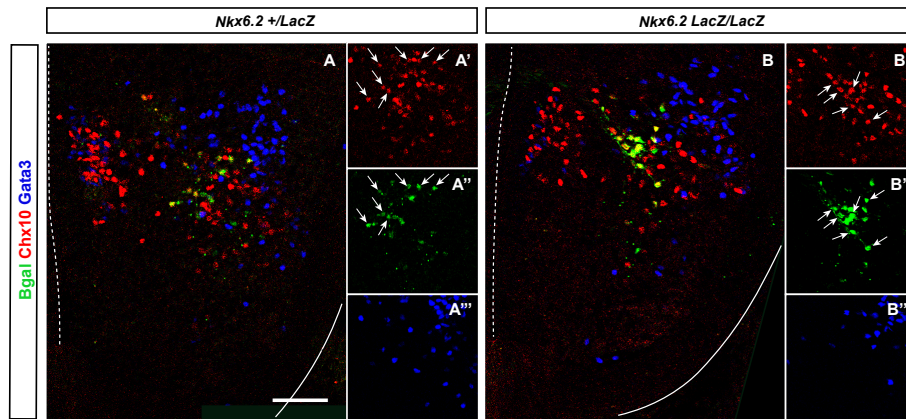
The analysis of the microarray comparison of control and of *Oc* mutant spinal cord transcriptome that we performed at e11.5 allowed us to select another potential regulator of the V2 diversification and/or migration: *Nkx6.2*. Although it was not significantly downregulated (0.7-fold decrease) in the microarray results, *Nkx6.2* is expressed in the p1 progenitor domain where it is known to control the V1 cell fate specification and the MN production in combination with *Nkx6.1*. In addition, *Nkx6.2* is expressed in postmitotic INs in the ventral region of the spinal cord and in a motor pool of the LMCI. To determine whether *Nkx6.2* could control the V2 differentiation and/or migration, we first characterized its expression pattern in the ventral region of the spinal cord by *in situ* hybridization on sections from control or *Hnf6/Oc2* compound mutant embryos. In control embryos, *Nkx6.2* transcripts were detected in progenitors (arrows in Figure 42A-C), in postmitotic INs (red circles in Figure 42A-C) and only at brachial and thoracic regions in subsets of LMC neurons (black circles in Figure 42A-C). In the absence of OC factors, *Nkx6.2* was still expressed in the progenitor domain (arrows in Figure 42D-F). Surprisingly, its expression was lost in the postmitotic INs (asterisks in Figure 42D-1F), while it was expanded in LMC area at both brachial and lumbar levels (black circles in Figure 42D-F). These results indicate that OC factors control the *Nkx6.2* expression differentially in INs and in MNs.



**Figure 42: OC factors control the expression of *Nkx6.2*.** (A-F) *In situ* hybridization labelings on transverse sections at each level of control or *Hnf6*<sup>-/-</sup>;*Oc2*<sup>-/-</sup> spinal cords at e11.5. (A-C) In control embryos, *Nkx6.2* is expressed in progenitors (arrows), in postmitotic INs (red circles), and in a motor subset of the LMCI at brachial and at lumbar levels (black circles). (D-F) In the OC mutant embryos, *Nkx6.2* is still present in the progenitor domain (arrows). Its expression is lost in the postmitotic INs (asterisks), while it is expanded in the LMC neurons at both brachial and lumbar levels (black circles).

### ***Nkx6.2* is expressed in the V2a interneurons**

In addition to the p1 domain and to the LMC neurons, *Nkx6.2* was present in INs in the ventral region of the spinal cord near to the V2 IN area. To determine whether *Nkx6.2* was expressed in V2 populations, we studied the *Nkx6.2* expression pattern in *Nkx6.2* transgenic mice wherein LacZ is expressed under the control of *Nkx6.2* regulating sequences (Southwood et al., 2004). In heterozygous *Nkx6.2*<sup>+/-LacZ</sup> embryos, although the β-galactosidase signal was weak, β-galactosidase was present in few cells expressing *Chx10* and therefore corresponded to a small subset of V2a INs (arrows in Figure 43A-A''). Indeed, β-galactosidase was not present in the Gata3+ V2b cells or other IN populations (Figure 43A-A'' and data not show). In *Nkx6.2*-null mutant, the β-galactosidase signal was stronger as the 2 alleles express LacZ. β-galactosidase was co-detected with *Chx10* in some V2a INs (arrows in Figure 43B-B''). Thus, these data demonstrate *Nkx6.2* is expressed in a subpopulation of V2a postmitotic INs.



**Figure 43: *Nkx6.2* is expressed in the V2a interneurons.** Immunolabelings on transverse brachial spinal cord sections of *Nkx6.2*<sup>+/LacZ</sup> heterozygous or *Nkx6.2*<sup>LacZ/LacZ</sup> mutant embryos at e12.5. **(A)** In heterozygous *Nkx6.2*<sup>+/LacZ</sup> embryos,  $\beta$ -galactosidase is present in few cells that express *Chx10* in some V2a interneurons (arrows), while it is not present in Gata3+ V2b cells. **(B)** Similar observations are made in the *Nkx6.2*-null embryos (arrows). Scale bar = 50  $\mu$ m.

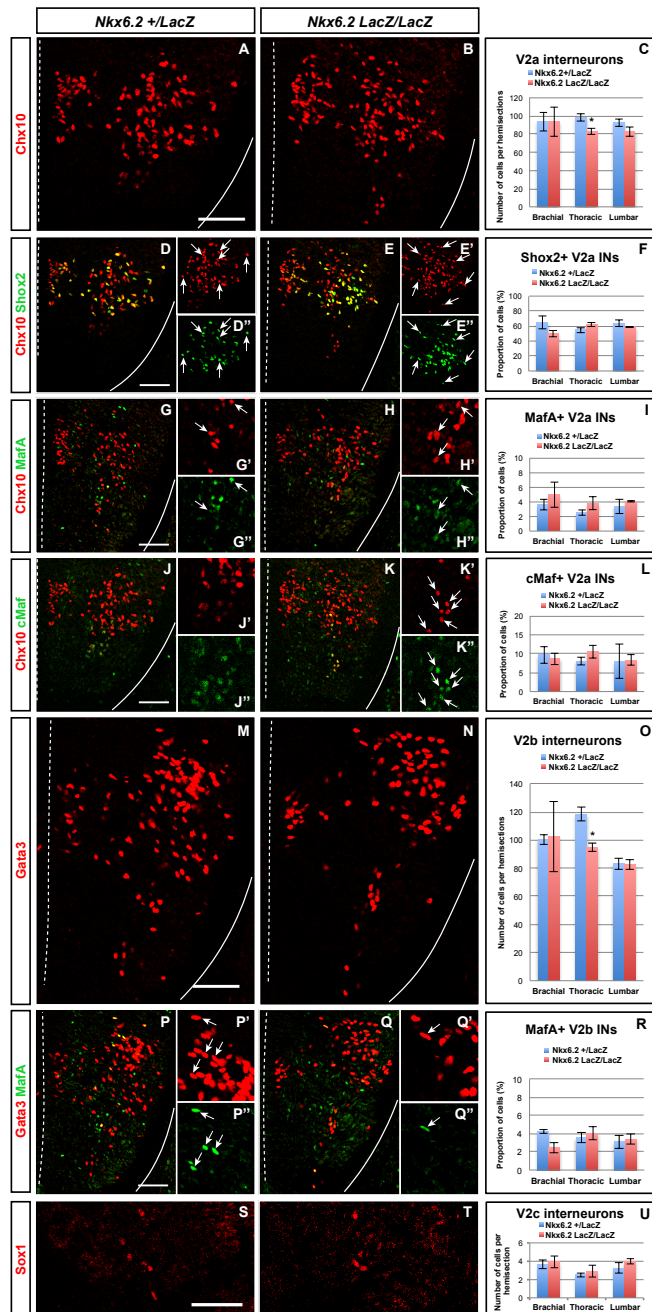
### **Nkx6.2 does not seem to regulate the V2 population diversification**

The expression of *Nkx6.2* in the V2a INs suggested that it could regulate V2 diversification and/or migration. To study the role of *Nkx6.2* during V2 INs diversification, we characterized the phenotype of different populations and subpopulations of V2 in *Nkx6.2*-null mutant at e12.5 (n=3). As the phenotype of control and heterozygous *Nkx6.2*<sup>+/LacZ</sup> embryos were comparable we used the heterozygous as control. In the absence of *Nkx6.2*, the total number of Chx10+ V2a INs was significantly, although modestly, decreased at thoracic level, while it was not the case at brachial and thoracic levels as compared to control embryos (Figure 44A-C). Among the V2a subpopulations studied, none of the Shox2+, MafA+ or cMaf+ subsets were altered in *Nkx6.2* mutant embryos (Figure 44D-L). Hence, *Nkx6.2* is not required for the diversification of the V2a INs but seems to be required for proper production at thoracic level of the spinal cord.

To determine whether the decrease of V2a INs was compensated by an increase of V2b INs at thoracic level, we also characterized the phenotype of V2b

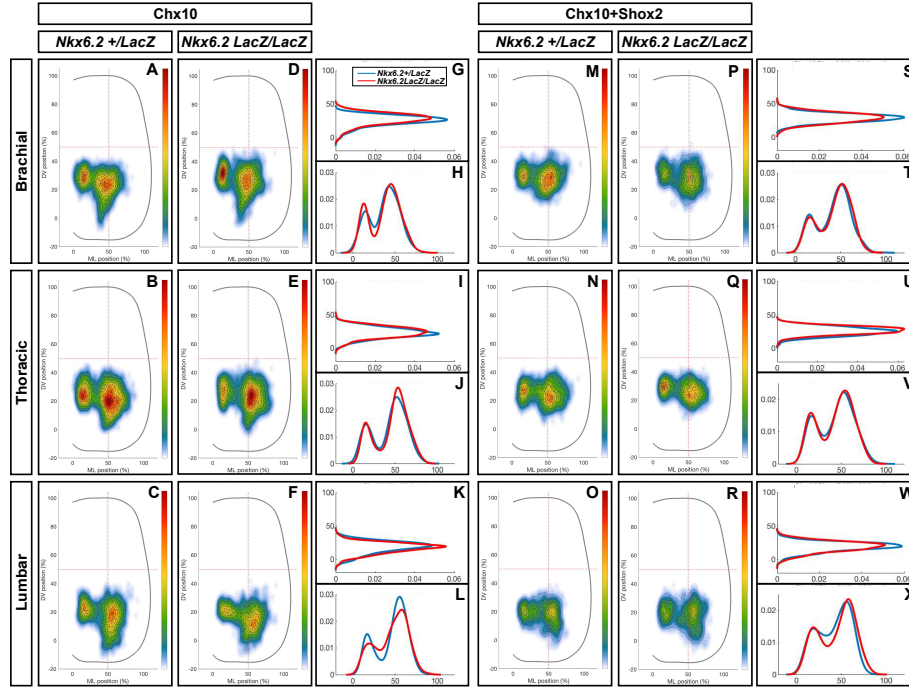
INs in the absence of *Nkx6.2*. Surprisingly, as observed for the V2a INs, the number of Gata3<sup>+</sup> V2b INs was significantly decreased at thoracic level, while the number of those cells was unchanged in the other regions of the spinal cord (Figure 44M-O). In addition, the MafA<sup>+</sup> V2b cells were present in comparable number in *Nkx6.2* mutant embryos (Figure 44P-R). Finally, the V2c population was not affected in the absence of *Nkx6.2*. Taken together, our data suggest that *Nkx6.2* controls the production of V2a and V2b INs at thoracic level at e12.5 but not the V2c production or the diversification of these populations. However, we cannot rule out that the slight decrease of V2a and V2b INs was a possible artifact due to the individual variability between embryos.

## Results



**Nkx6.2 controls the distribution of V2 interneurons**

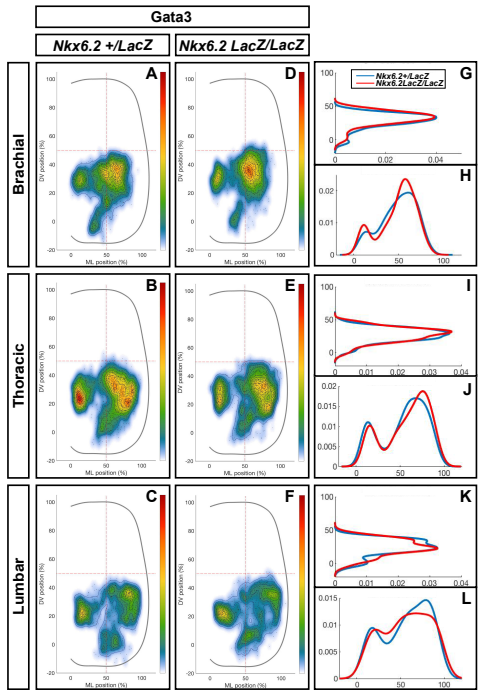
Careful examination of immunofluorescence labelings suggested that Nkx6.2 might contribute to the regulation of V2 IN distribution. To assess whether Nkx6.2 regulate the migration of V2 INs, we next performed a quantitative analysis of the distribution of each V2 IN population and subpopulation in *Nkx6.2* mutant mice. However, the MafA<sup>+</sup> and cMaf<sup>+</sup> subpopulations do not allow quantitative analysis of their distribution due to their small size. In heterozygous *Nkx6.2*<sup>+/LacZ</sup> embryos, V2a INs are distributed in 2 connected clusters, a major central group and a minor medial group, at each level of the spinal cord (Figure 45A-O). In mutant embryos, the relative cell distribution between the 2 clusters seemed altered (Figure 45D-L). Indeed, at the brachial level, we observed more segregated clusters with more cells in the medial group (Figure 45D,G-H); at thoracic level, slightly more cells in the central group (Figure 45E,I-J); finally, at lumbar level, clusters were less separated (Figure 45F,K-L). Subtle changes were also observed for the Shox2<sup>+</sup> V2a subpopulation (Figure 45M-X). In heterozygous *Nkx6.2*<sup>+/LacZ</sup> embryos this subpopulation is distributed in a minor medial and a major lateral cluster. In *Nkx6.2*-null mutant embryos, the 2 clusters were more segregated at lumbar level (Figure 45P-X). Hence, Nkx6.2 seems to contribute to the regulation of V2a migration.



**Figure 45: Nkx6.2 contributes to the regulation of the V2a distribution.** Distribution of V2a interneurons on transverse sections of the spinal cord in *Nkx6.2*<sup>+/LacZ</sup> heterozygous or *Nkx6.2*<sup>LacZ/LacZ</sup> mutant embryos at brachial, thoracic or lumbar level (only the right hemisection is shown). Two-dimension distribution graphs (left) show integration of cell distribution from multiple sections of multiple embryos of each genotype. One-dimension graphs (right) compare density distribution in control (blue) and in mutant embryos (red) on the dorso-ventral (upper) or the medio-lateral (lower) axis of the spinal cord. **(A-C)** In heterozygous *Nkx6.2*<sup>+/LacZ</sup> embryos, V2a interneurons are distributed in 2 connected clusters, a major central group and a minor medial group, at each level of the spinal cord. **(D-L)** In mutant embryos, the relative cell distribution between the 2 clusters seems altered, **(D,G-H)** with more separated clusters and more cells in the medial group at brachial, **(E,I-J)** more cells in the central group at thoracic level, and **(F,K-L)** less cells in both clusters that are less separated at lumbar level ( $n=3$ ,  $p \leq 0.001$  at brachial and thoracic levels,  $p \leq 0.05$  at lumbar level). **(M-O)** In heterozygous *Nkx6.2*<sup>+/LacZ</sup> embryos, Shox2+ V2a subpopulation is distributed in a minor medial and a major lateral clusters. In *Nkx6.2*-null mutant embryos, the differences are subtler with more separated clusters at lumbar level ( $n=3$ ,  $p=0.5112$  at brachial level,  $p \leq 0.001$  at thoracic level,  $p \leq 0.01$  at lumbar level).



We also assessed the distribution of the V2b in the absence of Nkx6.2. In heterozygous *Nkx6.2<sup>+/-</sup>LacZ* embryos, V2b cells were distributed in a major lateral cluster with minor subsets located medially or ventrally (Figure 46A-C). In the absence of Nkx6.2, as observed for the V2a population, the distribution between the 3 clusters was slightly altered (Figure 46D-L). The lateral cluster was more dense at brachial level (Figure 46D-E,G-J), while it was less dense at thoracic level (Figure 46F, K-L). Taken together, these data suggest that Nkx6.2 contributes to the regulation of both V2a and V2b population distribution at e12.5.



**Figure 46: Nkx6.2 contributes to the regulation of the V2b distribution.** (A-C) At e12.5 in heterozygous *Nkx6.2<sup>+/-</sup>LacZ* embryos, V2b cells are distributed in a major lateral cluster with minor subsets located medially or ventrally. (D-L) In the absence of Nkx6.2, the distribution between the 3 clusters is slightly altered, with more cells in the lateral cluster at brachial level and less cells in this cluster at thoracic level (n=3, p<0.001 at brachial and thoracic levels, p=0.7712 at lumbar level).

### **3.1.1. Discussion**

Our study demonstrates that the OC factors regulate *Nkx6.2* in a distinct manner in V2a INs and in MNs. Indeed, the characterization of *Nkx6.2* expression profile has shown that *Nkx6.2* is downregulated in the V2a population, while it is expanded in the LMC neurons in the absence of OC factors. We hypothesized that *Nkx6.2* expansion in the LMC is a consequence of the LMCI neuron production at the expense of the LMCm in OC-deficient embryos. These transcription factors being described as transcriptional activators, this suggests that the OC factors repress indirectly the *Nkx6.2* expression in the majority of the LMC neurons, as they do for *Pou2f2*. This raises the possibility that OC factors regulate *Nkx6.2* expression in a cell-context dependent manner by interacting with other factors or co-activators in the V2a INs that are not present in the MNs. Indeed, transcription factors can act differently in association with distinct complexes. Consistently, *Lhx3* consolidates the V2a or the MN identity by associating with other proteins in a tetramer or a hexamer complex, respectively.

The characterization of the *Nkx6.2* transgenic mice, in which the open reading frame of the first and the second exons have been replaced by a LacZ-PGKneo cassette, unveiled that *Nkx6.2* is expressed in a subset of V2a INs. Surprisingly, we showed that *Nkx6.2* seems to regulate the production of the V2a and the V2b INs at the thoracic level of the spinal cord, while their production is not affected in OC-deficient embryos. However, *Nkx6.2* neither regulates the size of the *Shox2*<sup>+</sup>, the *MafA*<sup>+</sup> or the *cMaf*<sup>+</sup> V2a subpopulations nor the *MafA*<sup>+</sup> V2b subpopulation. These observations suggest that the small reduction of V2a and V2b IN cell number at thoracic level could be an artifact due to an individual variability between embryos.

We also showed that *Nkx6.2* seems to regulate the V2a and V2b IN distribution. Although subtle, the differences observed after the quantitative

analysis of V2a and V2b IN distribution in the absence of *Nkx6.2* were significant. The distribution defects observed in the V2 populations are not due to the absence of the V2a subpopulation normally expressing *Nkx6.2*, as  $\beta$ -galactosidase is still present in V2a INs. However, *Nkx6.2* being only expressed in a small V2a subpopulation raises 2 possibilities: the whole V2a and V2b INs are mislocated as a consequence of V2a population migration defects or due to a non-cell autonomous role of *Nkx6.2*. These 2 hypotheses require further investigations. Similarly, OC factors regulate in a non-autonomous manner the maintenance of the LC during development, possibly via the rhombencephalic portion of the MTN, which is located in close vicinity of the LC and is absent in *Oc* mutants (Espana and Clotman, 2012a). Whether *Nkx6.2* also controls the MN diversification and their axonal connectivity remains to be investigated.

## **4. General discussion**

During the development of the spinal cord, several populations of interneurons are produced from 10 distinct progenitor domains. These populations have been extensively characterized and differ from each other in their molecular identity, migration, function, or input and output connectivity (Goulding, 2009). The majority of IN populations produced from the most dorsal progenitors (except for the dl3 and dl6 INs implicated in motor coordination) convey sensory informations from the periphery towards MNs and INs at different levels of the spinal cord or to the brain. In contrast, the IN populations that derive from the most ventral progenitor domains modulate motor activities by regulating MN activity. Recent studies demonstrated that each ventral population diversifies into subpopulations that ensure the flexibility and the versatility of the locomotor CPGs (Bikoff et al., 2016; Borowska et al., 2015; Borowska et al., 2013; Gosgnach et al., 2017; Hayashi et al., 2018; Talpalar et al., 2013). Beside diversification, other studies highlighted that the IN position is a relevant determinant of neuronal circuit organization (Bikoff et al., 2016; Goetz et al., 2015; Hayashi et al., 2018; Hilde et al., 2016; Surmeli et al., 2011; Sweeney et al., 2018). However, the underlying molecular or cellular mechanisms that control the diversification and the migration of spinal neuronal populations remain poorly investigated.

In this work, we provided evidence that each ventral IN population diversifies into multiple subpopulations and settle differently along the antero-posterior axis of the spinal cord. In addition, we demonstrated that the OC factors regulate the diversification of the V2a INs. Finally, we uncovered genetic cascades involving OC factors and their downstream targets *Pou2f2* or *Nkx6.2*, which control the distribution of V2 populations during development.

#### **4.1. The ventral IN diversification**

The motor behavior is a complex system that comprises apparently simple motor tasks such as walking, running, swimming and sophisticated movements like dexterous hand movements, climbing, dancing. Although a seemingly simple behavior like switching from walking to running looks quite easy, the organization of underlying spinal circuits to execute this motor task is complex and requires the recruitment of several neuronal populations. This suggests that the ventral neuronal populations, organized in local CPGs, are highly diversified to contribute to the modularity and the flexibility of diverse motor tasks (Arber, 2012; Gosgnach et al., 2017; Griener et al., 2015).

The execution of a specific motor task requires the activation of specific MNs in the spinal cord in order to regulate the contraction of adequate muscles. In the mammalian spinal cord, the diversification of MNs has been well characterized. They are organized and regionalized in motor columns reflecting their locomotor function. MNs innervating the limbs are gathered in LMCI and LMCm, MNs innervating the body wall musculature or the dorsal axial musculature are assembled in HMC and MMC, respectively, and finally, MNs in PGC innervate the sympathetic ventral chain. MNs in LMCs further diversify into motor pools that innervate individual muscles (Dasen and Jessell, 2009a; Francius and Clotman, 2014; Philippidou and Dasen, 2013). Until today, no motor pool has been identified in other motor columns. However, CPGs are present all along the antero-posterior axis of the spinal cord suggesting that thoracic and MMC motor columns also diversify into motor pools to integrate local CPGs and to ensure the specific innervation of individual muscles. The diversification of MNs reflects the non-redundant character of their motor outputs. The ventral IN populations being the neurons that modulate MN activity, this suggests that they extensively diversify to properly regulate locomotor functions. Although ventral IN

diversification had been poorly investigated, a recent study uncovered that V1 INs can be divided into ~50 subpopulations based on the combined expression of 19 transcription factors but also on their electrophysiological properties (Bikoff et al., 2016). Moreover, V2a INs are divided into type I and type II populations involved in the control of left-right alternation and in the target reaching phase of forelimbs, respectively. These 2 groups are also fractioned, at birth, into 11 subpopulations characterized by the combinatorial expression of transcription factors (Hayashi et al., 2018). It appears that these subpopulations are also regionally distributed along the antero-posterior axis of the spinal cord (Hayashi et al., 2018; Sweeney et al., 2018). These observations further support the fact that INs and MNs diversify and topographically distribute along the body axis to form local CPGs that ensure specific motor tasks.

In the present work, we showed that ventral IN populations can be subdivided into smaller subsets characterized by the differential expression of transcription factors. However, only few transcription factors highlighted in this study are included among the markers that fractionate the V1 and V2a IN populations highlighted by Bikoff et al., 2016 and Hayashi et al., 2018. It will be interesting to further investigate the correspondence between these subpopulations by co-immunofluorescence labelings. The existence of these subpopulations, as small as they are, suggests that they exert specific roles to finely regulate the locomotor coordination by directly projecting to MNs or indirectly via other IN populations. Furthermore, genetic ablation of identified transcription factors or cells in which they are expressed could be used to characterize the role of these neuronal subpopulations in the locomotor control by analyzing the motor behavior of these mutant mice. Finally, axonal retrograde labelings of outputs and primary afferent inputs would allow characterization of the microcircuit organization of each identified subpopulation (Bourane et al., 2015).

This diversity could also reflect the various sources of afferent inputs from supraspinal neurons. The activity of MNs is regulated by several supraspinal neuronal systems. Indeed, multiple descending pathways from different regions in the brainstem and the cortex modulate the involuntary, like emotional motor behavior, and voluntary, like head, trunk and limb movements, by projecting towards intermediate INs or directly to MNs (Lemon, 2008). Knowing that only few of them directly contact specific motor pools, the corticospinal projections could selectively project on intermediate IN subpopulations that innervate and recruit specific MNs for particular motor tasks. In the cervical region of the spinal cord, the type II V2 INs, for instance, are propriospinal neurons with bifurcating axonal trajectory: ascending axons to the lateral reticular nucleus and descending axons to specific MNs. These relay INs regulate the target reaching phase of the forelimbs (Arber, 2012; Azim et al., 2014; Hayashi et al., 2018). Indeed, the reaching task is composed of 3 steps: the reaching phase, in which the forelimbs approach the object, the grasp phase, in which the paws retrieve the object, and the digit abduction phase, during the grasp phase. Selective ablation of cervical V2a results in the specific defect of the target reaching phase, while the other phases remain unaffected (Azim et al., 2014).

From an evolutionary point of view, the diversification of spinal IN populations could reflect the complexity of the locomotor tasks in mammals. Beside the conservation of key patterning genes, important evolutionary modifications of neuronal circuit diversification and architecture occur in the vertebrates. Indeed, vertebrate evolution is accompanied by a passage from aquatic to terrestrial life and limb acquisition. Therefore, the transition from a basic undulatory to a limb-based locomotion requires a precise coordination of individual muscles mediated by complex CPGs in the ventral spinal cord (Jung and Dasen, 2015)

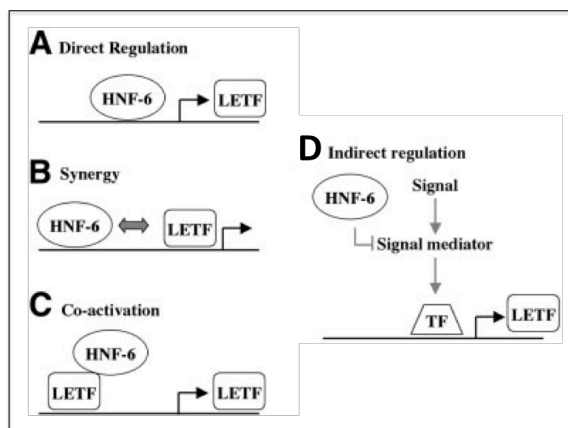
## **4.2. The OC factors control the V2a IN diversification**

Beyond the fact that transcription factors are used as specific markers of distinct cell types, their central role is to modulate the expression of genes that direct specification and functional properties of those cell types (Lee and Pfaff, 2001). Although the regulation of spinal population identity has been extensively studied within the spinal cord (Briscoe and Small, 2015; Lu et al., 2015), the transcriptional networks that regulate their diversification remain elusive. The regulators of the MN diversification have been well characterized. Indeed, the Hox gene family and Foxp1 mediates on the one hand the organization of MN in columns along the antero-posterior axis of the spinal cord and on the other hand the distribution of those columns and of motor pools along the dorso-ventral axis of the spinal cord (Philippidou and Dasen, 2013). Isl1 and Isl2 contribute to the regulation of the segregation between somatic and visceral MNs. In addition, Isl1 promotes the identity of LMCm neurons, while Lhx1 determines the LMCI identity. Lhx3 promotes the MMC neurons identity. Finally, OC factors promote the LMCm and the somatic neuron identities by controlling *Isl1* expression. Regarding the diversification of the ventral IN populations (Bikoff et al., 2016; Francius et al., 2013; Hayashi et al., 2018), it is not clear whether the characterized transcription factors could regulate their diversification into subpopulations

Within the V2a populations, we showed that the OC factors control the diversification of the MafA<sup>+</sup> and cMaf<sup>+</sup> V2a subpopulations. However, we do not know whether the OC factor directly control the expression of MafA and cMaf. In *Oc* mutant embryos, MafA and cMaf are still present in other populations. We showed that the MafA<sup>+</sup> V2b subpopulation is not affected in the absence of OC factors. This raises 2 possibilities: either OC factors control the expression of MafA and cMaf in a context-dependent manner or they control the generation of these 2 V2a subpopulations. These observations are consistent with previous works



conducted in our laboratory, which demonstrated that the OC factors directly stimulate *Isl1* expression in the LMCm and PGC neurons but not in the HMC and MMC somatic MNs, or in the dorsal population dl3 (Kabayiza et al., 2017; Roy et al., 2012). Similarly, OC control *Foxp1* expression in PGC cells but not in LMC cells (Roy et al., 2012). As in MNs, the transcriptional activity of OC factors seems to be context-dependent during the diversification of the V2 populations. Several studies highlighted 4 mechanisms by which HNF-6 modulates the expression of genes in the embryonic liver. First of all, HNF-6 directly binds and stimulates the *Hnf1 $\beta$*  promoter during the differentiation of hepatoblasts (Figure 47A). Secondly, HNF-6 synergistically stimulates the transcription of *Hnf4 $\alpha$*  with HNF-1 $\alpha$  (Hatzis and Talianidis, 2002), or that of the catalytic subunit of *glucose-6-phosphatase* gene in the hepatoplasts by interacting with HNF-4 and recruiting its PGC-1 $\alpha$  co-factor (Figure 47B) (Beaudry et al., 2006). Thirdly, HNF-6 does not directly bind to the *Foxa2* promoter to stimulate its transcription but acts as a transcriptional coactivator by interacting with FoxA2 and recruiting the CBP and p300/pCAF cofactors (Figure 47C) (Rausa et al., 1997). Finally, HNF-6 indirectly inhibits *Foxa1* stimulation by TGF $\beta$  signaling, probably by modulating other transcription factors (Figure 47D) (Clotman et al., 2005; Plumb-Rudewiez et al., 2004).



**Figure 47: Mechanisms of HNF-6 transcriptional activity (Plumb-Rudewiez et al., 2004).** In the liver, HNF-6 regulates the transcription of specific genes according to 4 distinct mechanisms: direct regulation (A), synergic regulation (B), co-activation (C), and indirect regulation (D). LETf = Liver-enriched transcription factor, TF = transcription factor.

Our data suggest that cofactors could be present in V2a INs, but not in V2b cells, to modulate either the OC binding on these genes or the OC activity. Further experiments will be necessary to elucidate whether OC factors directly bind to the MafA or cMaf promoter. Then, a deep analysis of the OC cofactors in the CNS will allow a better comprehension of the diversification mechanisms regulated by the OC factors.

We also showed that the production of V2c INs is delayed in OC mutant embryos, which suggests that the OC factors contribute to their production from the V2b population.

#### **4.3. The OC factors control the V2 IN distribution**

During CNS development, thousands of neuronal populations are produced and undertake cellular migration and axonal pathfinding to adequately integrate neuronal circuits. In the cortex, the excitatory INs migrate radially from the ventricular zone to the pial surface in order to organize in an inside-out manner in 6 layers and form the cortical plate. The inhibitory IN migrate tangentially from the ganglion eminences then migrate radially to integrate the cortical plate (Huang, 2009). In the spinal cord, the progenitor cells migrate radially in the ventricular zone before cell cycle exit. Afterwards, differentiating neuronal populations continue to migrate either radially along the medio-lateral axis or tangentially along the dorso-ventral axis. Neuronal populations also migrate along the antero-posterior axis, which is likely important to form proper local CPGs and MN connections with particular target muscles (Chen, 2018). Although the radial migration of MNs is controlled by Reelin signaling and their proper organization in motor pools is regulated by cadherins (Chen, 2018; Palmesino et al., 2010; Phelps et al., 2002; Yip et al., 2000), regulators of ventral INs migration are still unknown.

We uncovered that the OC factors control proper settling position of V2a, Shox2+ V2a and V2b populations, while they are produced in same cell numbers in *Oc* mutant than in control embryos. We hypothesize that even slight perturbation of their distribution could have influences on the microcircuit organization. Indeed, Hidle et al., 2016 demonstrated that *Satb2* defines an inhibitory dorsal IN population that relay sensory information to ventral INs and MNs. This population further diversifies into 2 subpopulations and *Satb2* is necessary for their proper localization on the mediolateral axis into the spinal cord. In *Satb2* mutants, mislocation of the *Satb2*+ neuron subpopulations results in input and output connectivity impairments, indicating that a specific localization is required for a proper integration of *Satb2*+ neurons in motor circuits. In addition, other studies have demonstrated that MN mislocation or switch of their identity results in wrong sensory input wiring. The monosynaptic sensory-motor connections between proprioceptive sensory neurons and motor neurons convey feedback from individual muscles to specific MNs that innervate the same muscles. These feedback connections are dorso-ventrally organized so that they correlate with the dorso-ventral MN positioning, i.e. the most ventral MNs innervate the most proximally-located muscles. In *Foxp1* mutants, although the MN position is altered, the proprioceptive sensory neurons project to the appropriate spatial area regardless of the identity of their MN targets, resulting in microcircuit wiring defects (Surmeli et al., 2011). Furthermore, the columnar MN identity regulates the input connectivity from proprioceptive sensory neurons and premotor INs. In *Hoxc9* mutant mice, HMC neurons are replaced by limb LMC fate due to the derepression of *Hoxc6*. This MN identity switch results in an extension of limb-level proprioceptive sensory axons towards ectopic LMC neurons at thoracic level. Surprisingly, the ectopic LMC neurons project to the intercostal target muscles. Moreover, LMC neurons normally receive inputs from ventral

premotor INs predominantly located ipsilaterally to the target muscle, while HMC neurons receive inputs from both contralateral and ipsilateral premotor INs. In *Hoxc9* mutant mice, the ectopic LMC neurons receive predominantly inputs from ipsilateral premotor INs at thoracic level, indicating a change in the connectivity of the premotor INs (Baek et al., 2017).

We showed that both medial and lateral V2a clusters are reorganized at each level of the spinal cord with more cells in the medial group at the expense of the lateral group in *Oc* mutant embryos. These 2 clusters are thought to be functionally distinct. Indeed, the medial group is predominantly composed of locally-projecting type I V2a INs that are likely axial premotor INs present all along the antero-posterior axis of the spinal cord. The lateral group is composed of locally- and supraspinally-projecting V2a INs distributed in counter-gradients along the antero-posterior axis of the spinal cord and most likely distinctly control fore- and hindlimbs (Goetz et al., 2015; Hayashi et al., 2018). These data suggest that the absence of OC factors could disturb the type I and II V2a wiring within local CPGs with an increase of the locally-projecting axial premotor INs. Further experiments like axonal retrograde labelings by viral injection into the brainstem or specific muscles will be necessary to investigate the impact of the absence of OC factors on the local CPG wiring. However, a lot of spinal populations are affected in *Oc* mutant embryos making difficult the specific characterization of OC factors roles during the V2 development. That is why those analyzes should be done on conditional mutant for OC in the V2 or in the V2a populations. We have in our laboratory a transgenic mouse model in which we could inactivate the expression of OC factors in all the V2 INs, the *Vsx1-CreER<sup>T2</sup>* mouse line.

Beside neuronal connectivity, the locomotor coordination could also be altered by V2a population mislocation. Even if the locomotor defects would be

probably severe, the double mutants for Hnf6 and Oc2 die at birth making behavioral tests impossible to perform. Interestingly, simple Hnf6 mutant mice exhibit muscular weakness and abnormal positioning of hindlimbs, as well as balance and coordination defects (Audouard et al., 2013; Audouard et al., 2012). In the OC-deficient embryos, the reduction of the lateral group containing V2a that control limb coordination could lead to left-right alternation and target reaching phase defects observed when the V2a are lost (Azim et al., 2014; Crone et al., 2009). As mentioned above, a conditional mutant for OC in the V2 or V2a populations would allow to characterize the specific role of OC factors in the development of these populations and to evaluate the impact on the locomotor coordination. It should be noted that this approach would be necessary for several other spinal populations.

Although the V2b involvement in specific local CPGs has not been yet characterized, the alteration of the relative distribution between the 3 clusters suggests that microcircuit organization and locomotor coordination defects are likely to exist in the absence of OC factors. Therefore, a deep analysis of the V2b cluster connectivity would give insights to better characterize their contribution to specific microcircuits.

#### **4.4. OC factors control the expression of Pou2f2 and Nkx6.2**

In an effort to elucidate the molecular mechanisms and consequently identify genes downstream of OC that may contribute to IN differentiation and distribution, we performed a microarray comparison of control or OC-deficient spinal cord transcriptomes. Only few genes were differentially expressed in absence of OC factors. We should have probably dissected separately the ventral and the dorsal region of the spinal cord to increase the sensitivity of the

transcriptome comparison. Among these genes, *Pou2f2* was upregulated but not significantly (1.57-fold increase). We first characterized the *Pou2f2* expression profile in the spinal cord and confirmed its upregulation by *in situ* hybridization on sections of control or OC-deficient embryos. Then, we surprisingly uncovered 4 neuronal *Pou2f2* isoforms produced in the developing spinal cord. The first exon of these isoforms is distinct and is located ~47kb upstream of the transcription initiation site used in B-cell *Pou2f2* in the mouse genome, suggesting that they are under the control of an alternative promoter. We also showed that these spinal variants are regulated by OC factors with a higher efficacy than the 6 B-cell *Pou2f2* isoforms (2.6-fold increase versus 1.3-fold increase). The OC factors being characterized as transcriptional activators, this suggests that they indirectly repress *Pou2f2* expression. Chromatin immunoprecipitation experiments would allow us to assess the direct or indirect OC factor interaction on the alternative *Pou2f2* promoter.

We showed that the *Pou2f2*+ V2a and V2b subpopulations exhibit similar distribution alterations than the whole V2a and V2b populations in OC-deficient embryos. There is an increase of the medial group of the *Pou2f2*+ V2a subpopulation at the expense of the lateral group. This observation suggests that the *Pou2f2* upregulation in the V2 populations could be involved in the diversification and the migration defects discussed above for the whole V2a and V2b populations (Goetz et al., 2015; Hayashi et al., 2018).

We also uncovered that OC factors stimulate *Nkx6.2* expression in the V2a population while they repress its expression in the LMC MNs, as they do for *Pou2f2*. These observations explain the fact that *Nkx6.2* was not differentially expressed in the absence of OC factors in the microarray results (0.7-fold decrease). The regulation of *Nkx6.2* expression by OC factors seems to be context-dependent. This raises the possibility that OC factors regulate *Nkx6.2* expression

according to distinct mechanisms by associating with specific complex of transcription factors. Similarly, Lhx3 and NLI are associated with other proteins in tetramer and hexamer complexes to consolidate the V2a and MN cell fate, respectively (Clovis et al., 2016; Lee et al., 2008; Thaler et al., 2002).

#### **4.5. OC-Pou2f2 and OC-Nkx6.2 genetic cascades control the V2 distribution**

We demonstrated by loss- and gain-of-function experiments that Pou2f2 is part of a genetic cascade involved in the control of the V2 population distribution. Although the distribution defects do not fully correlate with the phenotype observed in the OC-deficient embryos, we cannot rule out that it would be the case at later developmental stages. Indeed, the experimental models and the embryonic stages were different. Moreover, the absence of similitude between the consequences of the Pou2f2 overexpression after chick spinal cord electroporation and the Pou2f2 upregulation in *Oc* mutant mouse embryos could be explained by the fact that expression levels are often higher after *in ovo* electroporation. Finally, loss- and gain-of-function experiments do not give systematically opposite effects, but most of the time, complementary data. Through these experiments, we however showed Pou2f2 contribution to the control of V2 IN distribution. According to our assumptions, Pou2f2 could promote the axial premotor V2a identity at the expense of the limb premotor V2a and could control their position on the medio-lateral axis of the spinal cord. Thus, its overexpression could provoke similar locomotor defects as in the absence of the OC factors, or more postural defects could be expected in the absence of Pou2f2. However, the *Pou2f2* mutant mice die at birth, making locomotor

behavior analyses impossible to perform at adult stage. Fictive locomotion studies could be considered to describe locomotor coordination defects in the absence of *Pou2f2*. Indeed, this method allows evaluating several characteristics of locomotor-like activity, namely: rhythm and speed, left-right alternation, and flexor-extensor alternation. However, we cannot exclude that the mislocation of dorsal IN populations in the *Pou2f2* mutant embryos (Masgutova G. et al., in preparation) does not impact on the organization of local CPGs. Therefore, a specific *Pou2f2* ablation in the V2 INs would enable to determine the specific impact of V2 mislocation on the locomotor function.

We also showed that *Nkx6.2* is part of a genetic program that regulates V2 IN distribution. *Nkx6.2* being expressed in a small subpopulation of V2a, distribution alterations observed in the whole V2a and V2b populations raise 2 hypotheses: either *Nkx6.2* controls the distribution of a V2a subpopulation and their mislocation in the spinal cord in the absence of *Nkx6.2* prevents other cells to settle at proper locations; or *Nkx6.2* regulates the distribution of the whole V2a and V2b populations in a non-cell autonomous manner. The second hypothesis seems to be the most likely and is consistent with other studies from our laboratory. Indeed, Espana et al., 2012 hypothesized that OC factors regulate in a non-autonomous manner the maintenance of the LC during development, possibly via the rhombencephalic portion of the MTN, which is located in close vicinity of the LC and is absent in *Oc* mutants. Moreover, conditional inactivation of OC factors in MNs seems to influence in part the differentiation and the distribution of ventral IN populations (Francius C., Hidalgo-Figueroa M., Toch M., Bozet M. and Clotman F., work in progress). In *Nkx6.2* mutant embryos, the development of LMC neurons seems to be also affected (Toch M., work in progress). Axonal retrograde labelings will be necessary to characterize the connectivity of V2a INs and MNs in the absence of *Nkx6.2*.



These observations lead to propose axon guidance molecules as downstream effectors of these newly characterized genetic cascades. These molecules are secreted or transmembrane proteins. They are, including Netrin and their DCC/Neogenin and UNC5 receptors, SLIT and their ROBO receptors, Semaphorins and their Neuropilin and Plexin receptors, Ephrin and their Eph receptors, chemokines and their receptors, or Cadherins. They constitute attractive or repellant cues that guide the axons and cell bodies of several populations in nervous system. In the spinal cord, they have been mainly characterized in commissural axon guidance of dorsal and ventral IN populations but also in MN axons guidance and MN soma positioning (Chen, 2018). Preliminary data in the laboratory indicate that the expression of the transmembrane *Sema6A* and its *PlxnA4* receptor is reduced in the ventral regions of the spinal cord in *OC*-deficient embryos. *Sema6A/PlxnA4* pathway is known to act as a repellant cue. The reduction of repellant cues, produced by cells that express *Nkx6.2*, *Pou2f2* or *Oc* factors, could result in distribution defects in the majority of the V2 populations. This could explain the non-cell autonomous regulation of *Nkx6.2* discussed above. A characterization of *Sema6A* and *PlxnA4* expression of in *Pou2f2* and *Nkx6.2* mutant embryos would be necessary to assess the role of these molecules in the control of the V2 IN migration.

## **5. Bibliography**

- Agalliu, D., Takada, S., Agalliu, I., McMahon, A.P., Jessell, T.M., 2009. Motor neurons with axial muscle projections specified by Wnt4/5 signaling. *Neuron* 61, 708-720.
- Agius, E., Soukkaieh, C., Danesin, C., Kan, P., Takebayashi, H., Soula, C., Cochard, P., 2004. Converse control of oligodendrocyte and astrocyte lineage development by Sonic hedgehog in the chick spinal cord. *Developmental biology* 270, 308-321.
- Al-Mosawie, A., Wilson, J.M., Brownstone, R.M., 2007. Heterogeneity of V2-derived interneurons in the adult mouse spinal cord. *Eur J Neurosci* 26, 3003-3015.
- Alvarez, F.J., Dewey, D.E., McMillin, P., Fyffe, R.E., 1999. Distribution of cholinergic contacts on Renshaw cells in the rat spinal cord: a light microscopic study. *J Physiol* 515 ( Pt 3), 787-797.
- Alvarez, F.J., Jonas, P.C., Sapir, T., Hartley, R., Berrocal, M.C., Geiman, E.J., Todd, A.J., Goulding, M., 2005. Postnatal phenotype and localization of spinal cord V1 derived interneurons. *J Comp Neurol* 493, 177-192.
- Ampatzis, K., Song, J., Ausborn, J., El Manira, A., 2014. Separate microcircuit modules of distinct v2a interneurons and motoneurons control the speed of locomotion. *Neuron* 83, 934-943.
- Andjelic, S., Gallopin, T., Cauli, B., Hill, E.L., Roux, L., Badr, S., Hu, E., Tamás, G., Lambolez, B., 2009. Glutamatergic Nonpyramidal Neurons From Neocortical Layer VI and Their Comparison With Pyramidal and Spiny Stellate Neurons. *Journal of Neurophysiology* 101, 641-654.
- Annweiler, A., Muller-Immergluck, M., Wirth, T., 1992. Oct2 transactivation from a remote enhancer position requires a B-cell-restricted activity. *Mol Cell Biol* 12, 3107-3116.
- Annweiler, A., Zwilling, S., Wirth, T., 1994. Functional differences between the Oct2 transactivation domains determine the transactivation potential of individual Oct2 isoforms. *Nucleic Acids Res* 22, 4250-4258.
- Arber, S., 2012. Motor circuits in action: specification, connectivity, and function. *Neuron* 74, 975-989.

Arimatsu, Y., Ishida, M., Kaneko, T., Ichinose, S., Omori, A., 2003. Organization and development of corticocortical associative neurons expressing the orphan nuclear receptor Nurr1. *J Comp Neurol* 466, 180-196.

Armstrong, D.M., 1986. Supraspinal contributions to the initiation and control of locomotion in the cat. *Prog Neurobiol* 26, 273-361.

Audouard, E., Schakman, O., Ginion, A., Bertrand, L., Gailly, P., Clotman, F., 2013. The Onecut transcription factor HNF-6 contributes to proper reorganization of Purkinje cells during postnatal cerebellum development. *Mol Cell Neurosci* 56, 159-168.

Audouard, E., Schakman, O., Rene, F., Huettl, R.E., Huber, A.B., Loeffler, J.P., 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.

Azim, E., Jiang, J., Alstermark, B., Jessell, T.M., 2014. Skilled reaching relies on a V2a propriospinal internal copy circuit. *Nature* 508, 357-363.

Baek, M., Pivetta, C., Liu, J.P., Arber, S., Dasen, J.S., 2017. Columnar-Intrinsic Cues Shape Premotor Input Specificity in Locomotor Circuits. *Cell Rep* 21, 867-877.

Barber, M., Pierani, A., 2016. Tangential migration of glutamatergic neurons and cortical patterning during development: Lessons from Cajal-Retzius cells. *Dev Neurobiol* 76, 847-881.

Baron, O., Forthmann, B., Lee, Y.W., Terranova, C., Ratzka, A., Stachowiak, E.K., Grothe, C., Claus, P., Stachowiak, M.K., 2012. Cooperation of nuclear fibroblast growth factor receptor 1 and Nurr1 offers new interactive mechanism in postmitotic development of mesencephalic dopaminergic neurons. *J Biol Chem* 287, 19827-19840.

Batista, M.F., Jacobstein, J., Lewis, K.E., 2008. Zebrafish V2 cells develop into excitatory CiD and Notch signalling dependent inhibitory VeLD interneurons. *Dev Biol* 322, 263-275.

- Beaudry, J.B., Pierreux, C.E., Hayhurst, G.P., Plumb-Rudewiez, N., Weiss, M.C., Rousseau, G.G., Lemaigre, F.P., 2006. Threshold levels of hepatocyte nuclear factor 6 (HNF-6) acting in synergy with HNF-4 and PGC-1alpha are required for time-specific gene expression during liver development. *Mol Cell Biol* 26, 6037-6046.
- Beguín, S., Crepel, V., Aniksztejn, L., Becq, H., Pelosi, B., Pallesi-Pocachard, E., Bouamrane, L., Pasqualetti, M., Kitamura, K., Cardoso, C., Represa, A., 2013. An epilepsy-related ARX polyalanine expansion modifies glutamatergic neurons excitability and morphology without affecting GABAergic neurons development. *Cereb Cortex* 23, 1484-1494.
- Benito-Gonzalez, A., Alvarez, F.J., 2012. Renshaw cells and Ia inhibitory interneurons are generated at different times from p1 progenitors and differentiate shortly after exiting the cell cycle. *J Neurosci* 32, 1156-1170.
- Berkowitz, A., 2001. Rhythmicity of spinal neurons activated during each form of fictive scratching in spinal turtles. *Journal of neurophysiology* 86, 1026-1036.
- Betley, J.N., Wright, C.V., Kawaguchi, Y., Erdelyi, F., Szabo, G., Jessell, T.M., Kaltschmidt, J.A., 2009. Stringent specificity in the construction of a GABAergic presynaptic inhibitory circuit. *Cell* 139, 161-174.
- Bikoff, J.B., Gabitto, M.I., Rivard, A.F., Drobac, E., Machado, T.A., Miri, A., Brenner-Morton, S., Famojure, E., Diaz, C., Alvarez, F.J., Mentis, G.Z., Jessell, T.M., 2016. Spinal Inhibitory Interneuron Diversity Delineates Variant Motor Microcircuits. *Cell* 165, 207-219.
- Blacklaws, J., Deska-Gauthier, D., Jones, C.T., Petracca, Y.L., Liu, M., Zhang, H., Fawcett, J.P., Glover, J.C., Lanuza, G.M., 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.
- Boije, H., Kullander, K., 2018. Origin and circuitry of spinal locomotor interneurons generating different speeds. *Curr Opin Neurobiol* 53, 16-21.
- Borowska, J., Jones, C.T., Deska-Gauthier, D., Zhang, Y., 2015. V3 interneuron subpopulations in the mouse spinal cord undergo distinctive postnatal maturation processes. *Neuroscience* 295, 221-228.

- Borowska, J., Jones, C.T., 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.
- Boscher, C., Mege, R.M., 2008. Cadherin-11 interacts with the FGF receptor and induces neurite outgrowth through associated downstream signalling. *Cellular signalling* 20, 1061-1072.
- Bourane, S., Grossmann, K.S., Britz, O., Dalet, A., Del Barrio, M.G., Stam, F.J., Garcia-Campmany, L., Koch, S., Goulding, M., 2015. Identification of a spinal circuit for light touch and fine motor control. *Cell* 160, 503-515.
- Briscoe, J., Ericson, J., 1999. The specification of neuronal identity by graded Sonic Hedgehog signalling. *Semin Cell Dev Biol* 10, 353-362.
- Briscoe, J., Novitch, B.G., 2008. Regulatory pathways linking progenitor patterning, cell fates and neurogenesis in the ventral neural tube. *Philos Trans R Soc Lond B Biol Sci* 363, 57-70.
- Briscoe, J., Small, S., 2015. Morphogen rules: design principles of gradient-mediated embryo patterning. *Development* 142, 3996-4009.
- Briscoe, J., Sussel, L., Serup, P., Hartigan-O'Connor, D., Jessell, T.M., Rubenstein, J.L., Ericson, J., 1999. Homeobox gene *Nkx2.2* and specification of neuronal identity by graded Sonic hedgehog signalling. *Nature* 398, 622-627.
- Britz, O., Zhang, J., Grossmann, K.S., Dyck, J., Kim, J.C., Dymecki, S., Gosgnach, S., Goulding, M., 2015. A genetically defined asymmetry underlies the inhibitory control of flexor-extensor locomotor movements. *Elife* 4.
- Brunelli, S., Innocenzi, A., Cossu, G., 2003. *Bhlhb5* is expressed in the CNS and sensory organs during mouse embryonic development. *Gene Expr Patterns* 3, 755-759.
- Burrill, J.D., Moran, L., Goulding, M.D., Saueressig, H., 1997. *PAX2* is expressed in multiple spinal cord interneurons, including a population of *EN1+* interneurons that require *PAX6* for their development. *Development* 124, 4493-4503.
- Cai, J., St Amand, T., Yin, H., Guo, H., Li, G., Zhang, Y., Chen, Y., Qiu, M., 1999. Expression and regulation of the chicken *Nkx-6.2* homeobox gene suggest its possible involvement in the ventral neural patterning and cell fate specification. *Dev Dyn* 216, 459-468.

- Camos, S., Gubern, C., Sobrado, M., Rodriguez, R., Romera, V.G., Moro, M.A., Lizasoain, I., Serena, J., Mallolas, J., Castellanos, M., 2014. Oct-2 transcription factor binding activity and expression up-regulation in rat cerebral ischaemia is associated with a diminution of neuronal damage in vitro. *Neuromolecular Med* 16, 332-349.
- Carpenter, E.M., 2002. Hox genes and spinal cord development. *Dev Neurosci* 24, 24-34.
- Catela, C., Shin, M.M., Dasen, J.S., 2015. Assembly and function of spinal circuits for motor control. *Annu Rev Cell Dev Biol* 31, 669-698.
- Cazalets, J.R., 2000. Organization of the spinal locomotor network in neonatal rat, in: Inc., H.p. (Ed.), *Neurobiology of spinal cord injury*. R. Kalb and S.M. Stritmatter, pp. 89-111.
- Cazalets, J.R., Borde, M., Clarac, F., 1995. Localization and organization of the central pattern generator for hindlimb locomotion in newborn rat. *J Neurosci* 15, 4943-4951.
- Chakrabarty, K., Von Oerthel, L., Hellemons, A., Clotman, F., Espana, A., Groot Koerkamp, M., Holstege, F.C., Pasterkamp, R.J., Smidt, M.P., 2012. Genome wide expression profiling of the mesodiencephalic region identifies novel factors involved in early and late dopaminergic development. *Biol Open* 1, 693-704.
- Chamberlain, C.E., Jeong, J., Guo, C., Allen, B.L., McMahon, A.P., 2008. Notochord-derived Shh concentrates in close association with the apically positioned basal body in neural target cells and forms a dynamic gradient during neural patterning. *Development* 135, 1097-1106.
- Chanut, F., 2006. Wnt sets the stage for spinal cord patterning in the chick. *PLoS Biol* 4, e280.
- Chelban, V., Patel, N., Vandrovcova, J., Zanetti, M.N., Lynch, D.S., Ryten, M., Botia, J.A., Bello, O., Tribollet, E., Efthymiou, S., Davagnanam, I., Group, S.S., Bashiri, F.A., Wood, N.W., Rothman, J.E., Alkuraya, F.S., Houlden, H., 2017. Mutations in NKX6-2 Cause Progressive Spastic Ataxia and Hypomyelination. *Am J Hum Genet* 100, 969-977.
- Chen, Z., 2018. Common cues wire the spinal cord: Axon guidance molecules in spinal neuron migration. *Semin Cell Dev Biol*.

- Ciani, L., Salinas, P.C., 2005. WNTs in the vertebrate nervous system: from patterning to neuronal connectivity. *Nat Rev Neurosci* 6, 351-362.
- Clerc, R.G., Corcoran, L.M., LeBowitz, J.H., Baltimore, D., Sharp, P.A., 1988. The B-cell-specific Oct-2 protein contains POU box- and homeo box-type domains. *Genes Dev* 2, 1570-1581.
- Clotman, F., Jacquemin, P., Plumb-Rudewiez, N., Pierreux, C.E., Van der Smissen, P., Dietz, H.C., Courtoy, P.J., Rousseau, G.G., Lemaigre, F.P., 2005. Control of liver cell fate decision by a gradient of TGF beta signaling modulated by Onecut transcription factors. *Genes Dev* 19, 1849-1854.
- Clotman, F., Lannoy, V.J., Reber, M., Cereghini, S., Cassiman, D., Jacquemin, P., Roskams, T., Rousseau, G.G., Lemaigre, F.P., 2002. The onecut transcription factor HNF6 is required for normal development of the biliary tract. *Development* 129, 1819-1828.
- Clovis, Y.M., Enard, W., Marinaro, F., Huttner, W.B., De Pietri Tonelli, D., 2012. Convergent repression of Foxp2 3'UTR by miR-9 and miR-132 in embryonic mouse neocortex: implications for radial migration of neurons. *Development (Cambridge, England)* 139, 3332-3342.
- Clovis, Y.M., Seo, S.Y., Kwon, J.S., Rhee, J.C., Yeo, S., Lee, J.W., Lee, S., Lee, S.K., 2016. Chx10 Consolidates V2a Interneuron Identity through Two Distinct Gene Repression Modes. *Cell Rep* 16, 1642-1652.
- Cooper, K.L., Leisenring, W.M., Moens, C.B., 2003. Autonomous and nonautonomous functions for Hox/Pbx in branchiomotor neuron development. *Developmental biology* 253, 200-213.
- Corcoran, L.M., Karvelas, M., Nossal, G.J., Ye, Z.S., Jacks, T., Baltimore, D., 1993. Oct-2, although not required for early B-cell development, is critical for later B-cell maturation and for postnatal survival. *Genes Dev* 7, 570-582.
- Corcoran, L.M., Koentgen, F., Dietrich, W., Veale, M., Humbert, P.O., 2004. All known in vivo functions of the Oct-2 transcription factor require the C-terminal protein domain. *J Immunol* 172, 2962-2969.

- Crone, S.A., Quinlan, K.A., Zagoraïou, L., Droho, S., Restrepo, C.E., Lundfald, L., Endo, T., Setlak, J., Jessell, T.M., Kiehn, O., Sharma, K., 2008. Genetic ablation of V2a ipsilateral interneurons disrupts left-right locomotor coordination in mammalian spinal cord. *Neuron* 60, 70-83.
- Crone, S.A., Viemari, J.C., Droho, S., Mrejeru, A., Ramirez, J.M., Sharma, K., 2012. Irregular Breathing in Mice following Genetic Ablation of V2a Neurons. *J Neurosci* 32, 7895-7906.
- Crone, S.A., Zhong, G., Harris-Warrick, R., Sharma, K., 2009. In mice lacking V2a interneurons, gait depends on speed of locomotion. *J Neurosci* 29, 7098-7109.
- Dalla Torre di Sanguinetto, S.A., Dasen, J.S., Arber, S., 2008. Transcriptional mechanisms controlling motor neuron diversity and connectivity. *Current opinion in neurobiology* 18, 36-43.
- Dasen, J.S., 2017. Master or servant? emerging roles for motor neuron subtypes in the construction and evolution of locomotor circuits. *Curr Opin Neurobiol* 42, 25-32.
- Dasen, J.S., De Camilli, A., Wang, B., Tucker, P.W., Jessell, T.M., 2008. Hox repertoires for motor neuron diversity and connectivity gated by a single accessory factor, FoxP1. *Cell* 134, 304-316.
- Dasen, J.S., Jessell, T.M., 2009a. Hox networks and the origins of motor neuron diversity. *Curr Top Dev Biol* 88, 169-200.
- Dasen, J.S., Jessell, T.M., 2009b. Hox networks and the origins of motor neuron diversity. *Current topics in developmental biology* 88, 169-200.
- Dasen, J.S., Liu, J.P., Jessell, T.M., 2003. Motor neuron columnar fate imposed by sequential phases of Hox-c activity. *Nature* 425, 926-933.
- Dasen, J.S., Tice, B.C., Brenner-Morton, S., Jessell, T.M., 2005. A Hox regulatory network establishes motor neuron pool identity and target-muscle connectivity. *Cell* 123, 477-491.
- De Marco Garcia, N.V., Jessell, T.M., 2008. Early motor neuron pool identity and muscle nerve trajectory defined by postmitotic restrictions in Nkx6.1 activity. *Neuron* 57, 217-231.



- Del Barrio, M.G., Taveira-Marques, R., Muroyama, Y., Yuk, D.I., Li, S., Wines-Samuelson, M., Shen, J., Smith, H.K., Xiang, M., Rowitch, D., Richardson, W.D., 2007. A regulatory network involving Foxn4, Mash1 and delta-like 4/Notch1 generates V2a and V2b spinal interneurons from a common progenitor pool. *Development* 134, 3427-3436.
- Dent, C.L., Latchman, D.S., 1991. The overlapping octamer/TAATGARAT motif is a high-affinity binding site for the cellular transcription factors Oct-1 and Oct-2. *Biochem J* 277 ( Pt 2), 541-545.
- Dent, C.L., Lillycrop, K.A., Estridge, J.K., Thomas, N.S., Latchman, D.S., 1991. The B-cell and neuronal forms of the octamer-binding protein Oct-2 differ in DNA-binding specificity and functional activity. *Mol Cell Biol* 11, 3925-3930.
- Dessaud, E., McMahon, A.P., Briscoe, J., 2008. Pattern formation in the vertebrate neural tube: a sonic hedgehog morphogen-regulated transcriptional network. *Development* 135, 2489-2503.
- Dessaud, E., Ribes, V., Balaskas, N., Yang, L.L., Pierani, A., Kicheva, A., Novitsch, B.G., Briscoe, J., Sasai, N., 2010. Dynamic assignment and maintenance of positional identity in the ventral neural tube by the morphogen sonic hedgehog. *PLoS Biol* 8, e1000382.
- Diez del Corral, R., Storey, K.G., 2004. Opposing FGF and retinoid pathways: a signalling switch that controls differentiation and patterning onset in the extending vertebrate body axis. *Bioessays* 26, 857-869.
- Dong, B., Zhao, F.Q., 2007. Expression of the Oct-2 transcription factor in mouse mammary gland and cloning and characterization of a novel Oct-2 isoform. *Cell Tissue Res* 328, 595-606.
- Dougherty, K.J., Kiehn, O., 2010. Firing and cellular properties of V2a interneurons in the rodent spinal cord. *J Neurosci* 30, 24-37.
- Dougherty, K.J., Zagoraïou, L., Satoh, D., Rozani, I., Doobar, S., Arber, S., Jessell, T.M., Kiehn, O., 2013. Locomotor rhythm generation linked to the output of spinal shox2 excitatory interneurons. *Neuron* 80, 920-933.
- Eccles, J.C., Fatt, P., Koketsu, K., 1954. Cholinergic and inhibitory synapses in a pathway from motor-axon collaterals to motoneurons. *J Physiol* 126, 524-562.

- Eccles, J.C., Fatt, P., Landgren, S., 1956. Central pathway for direct inhibitory action of impulses in largest afferent nerve fibres to muscle. *J Neurophysiol* 19, 75-98.
- Elkouby, Y.M., Frank, D., 2010. *Wnt/beta-Catenin Signaling in Vertebrate Posterior Neural Development*, San Rafael (CA).
- England, S., Batista, M.F., Mich, J.K., Chen, J.K., Lewis, K.E., 2011. Roles of Hedgehog pathway components and retinoic acid signalling in specifying zebrafish ventral spinal cord neurons. *Development (Cambridge, England)* 138, 5121-5134.
- Ericson, J., Rashbass, P., Schedl, A., Brenner-Morton, S., Kawakami, A., van Heyningen, V., Jessell, T.M., Briscoe, J., 1997. Pax6 controls progenitor cell identity and neuronal fate in response to graded Shh signaling. *Cell* 90, 169-180.
- Espana, A., Clotman, F., 2012a. Onecut factors control development of the Locus Coeruleus and of the mesencephalic trigeminal nucleus. *Mol Cell Neurosci* 50, 93-102.
- Espana, A., Clotman, F., 2012b. Onecut transcription factors are required for the second phase of development of the A13 dopaminergic nucleus in the mouse. *J Comp Neurol* 520, 1424-1441.
- Fedtsova, N.G., Turner, E.E., 1995. Brn-3.0 expression identifies early post-mitotic CNS neurons and sensory neural precursors. *Mechanisms of development* 53, 291-304.
- 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.
- Francius, C., Clotman, F., 2014. Generating spinal motor neuron diversity: a long quest for neuronal identity. *Cell Mol Life Sci* 71, 813-829.
- Francius, C., Harris, A., Rucchin, V., Hendricks, T.J., Stam, F.J., Barber, M., Kurek, D., Grosveld, F.G., Pierani, A., Goulding, M., Clotman, F., 2013. Identification of multiple subsets of ventral interneurons and differential distribution along the rostrocaudal axis of the developing spinal cord. *PLoS One* 8, e70325.

Francius, C., Hidalgo-Figueroa, M., Debrulle, S., Pelosi, B., Rucchin, V., Ronellenfitch, K., Panayiotou, E., Makrides, N., Misra, K., Harris, A., Hassani, H., Schakman, O., Parras, C., Xiang, M., Malas, S., Chow, R.L., Clotman, F., 2016. Vsx1 Transiently Defines an Early Intermediate V2 Interneuron Precursor Compartment in the Mouse Developing Spinal Cord. *Front Mol Neurosci* 9, 145.

Gabitto, M.I., Pakman, A., Bikoff, J.B., Abbott, L.F., Jessell, T.M., Paninski, L., 2016. Bayesian Sparse Regression Analysis Documents the Diversity of Spinal Inhibitory Interneurons. *Cell* 165, 220-233.

Gammill, L.S., Bronner-Fraser, M., 2003. Neural crest specification: migrating into genomics. *Nat Rev Neurosci* 4, 795-805.

Gannon, M., Ray, M.K., Van Zee, K., Rausa, F., Costa, R.H., Wright, C.V., 2000. Persistent expression of HNF6 in islet endocrine cells causes disrupted islet architecture and loss of beta cell function. *Development* 127, 2883-2895.

Garcia-Campmany, L., Stam, F.J., Goulding, M., 2010. From circuits to behaviour: motor networks in vertebrates. *Current opinion in neurobiology* 20, 116-125.

Gard, C., Gonzalez Curto, G., Frarma, Y.E., Chollet, E., Duval, N., Auzie, V., Aurade, F., Vigier, L., Relaix, F., Pierani, A., Causeret, F., Ribes, V., 2017. Pax3- and Pax7-mediated Dbx1 regulation orchestrates the patterning of intermediate spinal interneurons. *Dev Biol* 432, 24-33.

Gaspard, N., Vanderhaeghen, P., 2010. Mechanisms of neural specification from embryonic stem cells. *Curr Opin Neurobiol* 20, 37-43.

Gierl, M.S., 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 & development* 20, 2465-2478.

Goetz, C., Pivetta, C., Arber, S., 2015. Distinct limb and trunk premotor circuits establish laterality in the spinal cord. *Neuron* 85, 131-144.

Gosgnach, S., Bikoff, J.B., Dougherty, K.J., El Manira, A., Lanuza, G.M., Zhang, Y., 2017. Delineating the Diversity of Spinal Interneurons in Locomotor Circuits. *J Neurosci* 37, 10835-10841.

Gosgnach, S., Lanuza, G.M., Butt, S.J., Saueressig, H., Zhang, Y., Velasquez, T., Riethmacher, D., Callaway, E.M., Kiehn, O., Goulding, M., 2006. V1 spinal neurons regulate the speed of vertebrate locomotor outputs. *Nature* 440, 215-219.

---

## Bibliography

- Goulding, M., 2009. Circuits controlling vertebrate locomotion: moving in a new direction. *Nat Rev Neurosci* 10, 507-518.
- Goulding, M., Lamar, E., 2000. Neuronal patterning: Making stripes in the spinal cord. *Curr Biol* 10, R565-568.
- Griener, A., Zhang, W., Kao, H., Wagner, C., Gosgnach, S., 2015. Probing diversity within subpopulations of locomotor-related V0 interneurons. *Dev Neurobiol* 75, 1189-1203.
- Grillner, S., 2003. The motor infrastructure: from ion channels to neuronal networks. *Nature reviews. Neuroscience* 4, 573-586.
- Grillner, S., Jessell, T.M., 2009. Measured motion: searching for simplicity in spinal locomotor networks. *Curr Opin Neurobiol* 19, 572-586.
- Gross, M.K., Dottori, M., Goulding, M., 2002. Lbx1 specifies somatosensory association interneurons in the dorsal spinal cord. *Neuron* 34, 535-549.
- Grossmann, K.S., Giraudin, A., Britz, O., Zhang, J., Goulding, M., 2010a. Genetic dissection of rhythmic motor networks in mice. *Progress in brain research* 187, 19-37.
- Grossmann, K.S., Giraudin, A., Britz, O., Zhang, J., Goulding, M., 2010b. Genetic dissection of rhythmic motor networks in mice. *Prog Brain Res* 187, 19-37.
- Grothe, C., Timmer, M., Scholz, T., Winkler, C., Nikkhah, G., Claus, P., Itoh, N., Arenas, E., 2004. Fibroblast growth factor-20 promotes the differentiation of Nurr1-overexpressing neural stem cells into tyrosine hydroxylase-positive neurons. *Neurobiology of disease* 17, 163-170.
- Guo, J., Anton, E.S., 2014. Decision making during interneuron migration in the developing cerebral cortex. *Trends Cell Biol* 24, 342-351.
- Hatzis, P., Talianidis, I., 2002. Dynamics of enhancer-promoter communication during differentiation-induced gene activation. *Mol Cell* 10, 1467-1477.
- Hatzopoulos, A.K., Stoykova, A.S., Erselius, J.R., Goulding, M., Neuman, T., Gruss, P., 1990. Structure and expression of the mouse Oct2a and Oct2b, two differentially spliced products of the same gene. *Development* 109, 349-362.

Haworth, K.E., Latinkic, B., 2009. Expression of *Xenopus tropicalis* HNF6/Onecut-1. *Int J Dev Biol* 53, 159-162.

Hayashi, M., Hinckley, C.A., Driscoll, S.P., Moore, N.J., Levine, A.J., Hilde, K.L., Sharma, K., Pfaff, S.L., 2018. Graded Arrays of Spinal and Supraspinal V2a Interneuron Subtypes Underlie Forelimb and Hindlimb Motor Control. *Neuron* 97, 869-884 e865.

Hilde, K.L., Levine, A.J., Hinckley, C.A., Hayashi, M., Montgomery, J.M., Gullo, M., Driscoll, S.P., Grosschedl, R., Kohwi, Y., Kohwi-Shigematsu, T., Pfaff, S.L., 2016. *Satb2* Is Required for the Development of a Spinal Exteroceptive Microcircuit that Modulates Limb Position. *Neuron* 91, 763-776.

Hisaoka, T., Nakamura, Y., Senba, E., Morikawa, Y., 2010. The forkhead transcription factors, *Foxp1* and *Foxp2*, identify different subpopulations of projection neurons in the mouse cerebral cortex. *Neuroscience* 166, 551-563.

Hochstim, C., Deneen, B., Lukaszewicz, A., Zhou, Q., Anderson, D.J., 2008. Identification of positionally distinct astrocyte subtypes whose identities are specified by a homeodomain code. *Cell* 133, 510-522.

Hodge, L.K., Klassen, M.P., Han, B.X., Yiu, G., Hurrell, J., Howell, A., Rousseau, G., Lemaigre, F., Tessier-Lavigne, M., Wang, F., 2007. Retrograde BMP signaling regulates trigeminal sensory neuron identities and the formation of precise face maps. *Neuron* 55, 572-586.

Hodson, D.J., Shaffer, A.L., Xiao, W., Wright, G.W., Schmitz, R., Phelan, J.D., Yang, Y., Webster, D.E., Rui, L., Kohlhammer, H., Nakagawa, M., Waldmann, T.A., Staudt, L.M., 2016. Regulation of normal B-cell differentiation and malignant B-cell survival by *OCT2*. *Proc Natl Acad Sci U S A* 113, E2039-2046.

Holz, A., Kollmus, H., Ryge, J., Niederkofler, V., Dias, J., Ericson, J., Stoeckli, E.T., Kiehn, O., Arnold, H.H., 2010. The transcription factors *Nkx2.2* and *Nkx2.9* play a novel role in floor plate development and commissural axon guidance. *Development* 137, 4249-4260.

Hong, S.K., Kim, C.H., Yoo, K.W., Kim, H.S., Kudoh, T., Dawid, I.B., Huh, T.L., 2002. Isolation and expression of a novel neuron-specific onecut homeobox gene in zebrafish. *Mech Dev* 112, 199-202.

- Hu, J., Huang, T., Li, T., Guo, Z., Cheng, L., 2012. c-Maf is required for the development of dorsal horn laminae III/IV neurons and mechanoreceptive DRG axon projections. *J Neurosci* 32, 5362-5373.
- Huang, Z., 2009. Molecular regulation of neuronal migration during neocortical development. *Mol Cell Neurosci* 42, 11-22.
- Hultborn, H., Jankowska, E., Lindstrom, S., 1971. Recurrent inhibition of interneurons monosynaptically activated from group Ia afferents. *J Physiol* 215, 613-636.
- Ingraham, H.A., Flynn, S.E., Voss, J.W., Albert, V.R., Kapiloff, M.S., Wilson, L., Rosenfeld, M.G., 1990. The POU-specific domain of Pit-1 is essential for sequence-specific, high affinity DNA binding and DNA-dependent Pit-1-Pit-1 interactions. *Cell* 61, 1021-1033.
- Iyaguchi, D., Yao, M., Watanabe, N., Nishihira, J., Tanaka, I., 2007. DNA recognition mechanism of the ONECUT homeodomain of transcription factor HNF-6. *Structure* 15, 75-83.
- Jacob, J., Briscoe, J., 2003. Gli proteins and the control of spinal-cord patterning. *EMBO Rep* 4, 761-765.
- Jacquemin, P., Durviaux, S.M., Jensen, J., Godfraind, C., Gradwohl, G., Guillemot, F., Madsen, O.D., Carmeliet, P., Dewerchin, M., Collen, D., Rousseau, G.G., Lemaigre, F.P., 2000. Transcription factor hepatocyte nuclear factor 6 regulates pancreatic endocrine cell differentiation and controls expression of the proendocrine gene *ngn3*. *Mol Cell Biol* 20, 4445-4454.
- Jacquemin, P., Lannoy, V.J., O'Sullivan, J., Read, A., Lemaigre, F.P., Rousseau, G.G., 2001. The transcription factor *oncut-2* controls the microphthalmia-associated transcription factor gene. *Biochem Biophys Res Commun* 285, 1200-1205.
- Jacquemin, P., Lannoy, V.J., Rousseau, G.G., Lemaigre, F.P., 1999. OC-2, a novel mammalian member of the ONECUT class of homeodomain transcription factors whose function in liver partially overlaps with that of hepatocyte nuclear factor-6. *J Biol Chem* 274, 2665-2671.
- Jacquemin, P., Lemaigre, F.P., Rousseau, G.G., 2003a. The Onecut transcription factor HNF-6 (OC-1) is required for timely specification of the pancreas and acts upstream of Pdx-1 in the specification cascade. *Dev Biol* 258, 105-116.

- Jacquemin, P., Pierreux, C.E., Fierens, S., van Eyll, J.M., Lemaigre, F.P., Rousseau, G.G., 2003b. Cloning and embryonic expression pattern of the mouse *Onecut* transcription factor OC-2. *Gene Expr Patterns* 3, 639-644.
- Jaegle, M., Ghazvini, M., Mandemakers, W., Piirsoo, M., Driegen, S., Levavasseur, F., Raghoenath, S., Grosveld, F., Meijer, D., 2003. The POU proteins Brn-2 and Oct-6 share important functions in Schwann cell development. *Genes & development* 17, 1380-1391.
- Jankowska, E., 2001a. Spinal interneuronal systems: identification, multifunctional character and reconfigurations in mammals. *J Physiol* 533, 31-40.
- Jankowska, E., 2001b. Spinal interneuronal systems: identification, multifunctional character and reconfigurations in mammals. *The Journal of physiology* 533, 31-40.
- Jankowska, E., 2008. Spinal interneuronal networks in the cat: elementary components. *Brain Res Rev* 57, 46-55.
- Jarrar, W., Vauti, F., Arnold, H.H., Holz, A., 2015. Generation of a Nkx2.2(Cre) knock-in mouse line: Analysis of cell lineages in the central nervous system. *Differentiation* 89, 70-76.
- Jessell, T.M., 2000. Neuronal specification in the spinal cord: inductive signals and transcriptional codes. *Nat Rev Genet* 1, 20-29.
- Johnson, I.P., Sears, T.A., 1988. Ultrastructure of interneurons within motor nuclei of the thoracic region of the spinal cord of the adult cat. *Journal of anatomy* 161, 171-185.
- Jordan, L.M., Liu, J., Hedlund, P.B., Akay, T., Pearson, K.G., 2008. Descending command systems for the initiation of locomotion in mammals. *Brain Res Rev* 57, 183-191.
- Joshi, K., Lee, S., Lee, B., Lee, J.W., Lee, S.K., 2009. LMO4 controls the balance between excitatory and inhibitory spinal V2 interneurons. *Neuron* 61, 839-851.
- Jovanovic, K., Burke, R.E., 2004. Anatomical organization of motoneurons and interneurons in the mudpuppy (*Necturus maculosus*) brachial spinal cord: the neural substrate for central pattern generation. *Canadian journal of physiology and pharmacology* 82, 628-636.

- Jung, H., Dasen, J.S., 2015. Evolution of patterning systems and circuit elements for locomotion. *Dev Cell* 32, 408-422.
- Jung, H., Lacombe, J., Mazzoni, E.O., Liem, K.F., Jr., Grinstein, J., Mahony, S., Mukhopadhyay, D., Gifford, D.K., Young, R.A., Anderson, K.V., Wichterle, H., Dasen, J.S., 2010. Global control of motor neuron topography mediated by the repressive actions of a single hox gene. *Neuron* 67, 781-796.
- Kabayiza, K.U., 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.
- Karlsson, D., Baumgardt, M., Thor, S., 2010. Segment-specific neuronal subtype specification by the integration of anteroposterior and temporal cues. *PLoS Biol* 8, e1000368.
- Karunaratne, A., Hargrave, M., Poh, A., Yamada, T., 2002. GATA proteins identify a novel ventral interneuron subclass in the developing chick spinal cord. *Developmental biology* 249, 30-43.
- Kiehn, O., 2006. Locomotor circuits in the mammalian spinal cord. *Annu Rev Neurosci* 29, 279-306.
- Kim, E.J., Battiste, J., Nakagawa, Y., Johnson, J.E., 2008. *Ascl1* (*Mash1*) lineage cells contribute to discrete cell populations in CNS architecture. *Mol Cell Neurosci* 38, 595-606.
- Kim, N., Park, C., Jeong, Y., Song, M.R., 2015. Functional Diversification of Motor Neuron-specific *Isl1* Enhancers during Evolution. *PLoS Genet* 11, e1005560.
- Kimura, Y., Satou, C., Higashijima, S., 2008. V2a and V2b neurons are generated by the final divisions of pair-producing progenitors in the zebrafish spinal cord. *Development* 135, 3001-3005.
- Kjaerulff, O., Kiehn, O., 1996. Distribution of networks generating and coordinating locomotor activity in the neonatal rat spinal cord in vitro: a lesion study. *J Neurosci* 16, 5777-5794.
- 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.



- Konig, H., Pfisterer, P., Corcoran, L.M., Wirth, T., 1995. Identification of CD36 as the first gene dependent on the B-cell differentiation factor Oct-2. *Genes Dev* 9, 1598-1607.
- Kristie, T.M., LeBowitz, J.H., Sharp, P.A., 1989. The octamer-binding proteins form multi-protein--DNA complexes with the HSV alpha TIF regulatory protein. *EMBO J* 8, 4229-4238.
- Krumlauf, R., Marshall, H., Studer, M., Nonchev, S., Sham, M.H., Lumsden, A., 1993. Hox homeobox genes and regionalisation of the nervous system. *J Neurobiol* 24, 1328-1340.
- Kyriakatos, A., Mahmood, R., Ausborn, J., Porres, C.P., Buschges, A., El Manira, A., 2011. Initiation of locomotion in adult zebrafish. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 31, 8422-8431.
- Lai, H.C., Seal, R.P., Johnson, J.E., 2016. Making sense out of spinal cord somatosensory development. *Development* 143, 3434-3448.
- Lammi, J., Aarnisalo, P., 2008. FGF-8 stimulates the expression of NR4A orphan nuclear receptors in osteoblasts. *Molecular and cellular endocrinology* 295, 87-93.
- Landry, C., Clotman, F., Hioki, T., Oda, H., Picard, J.J., Lemaigre, F.P., Rousseau, G.G., 1997. HNF-6 is expressed in endoderm derivatives and nervous system of the mouse embryo and participates to the cross-regulatory network of liver-enriched transcription factors. *Dev Biol* 192, 247-257.
- Lannoy, V.J., Burglin, T.R., Rousseau, G.G., Lemaigre, F.P., 1998. Isoforms of hepatocyte nuclear factor-6 differ in DNA-binding properties, contain a bifunctional homeodomain, and define the new ONECUT class of homeodomain proteins. *J Biol Chem* 273, 13552-13562.
- Lannoy, V.J., Rodolosse, A., Pierreux, C.E., Rousseau, G.G., Lemaigre, F.P., 2000. Transcriptional stimulation by hepatocyte nuclear factor-6. Target-specific recruitment of either CREB-binding protein (CBP) or p300/CBP-associated factor (p/CAF). *J Biol Chem* 275, 22098-22103.
- Lanuza, G.M., Gosgnach, S., Pierani, A., Jessell, T.M., Goulding, M., 2004. Genetic identification of spinal interneurons that coordinate left-right locomotor activity necessary for walking movements. *Neuron* 42, 375-386.

- Latchman, D.S., 1996. The Oct-2 transcription factor. *Int J Biochem Cell Biol* 28, 1081-1083.
- Laudadio, I., Manfroid, I., Achouri, Y., Schmidt, D., Wilson, M.D., Cordi, S., Thorrez, L., Knoops, L., Jacquemin, P., Schuit, F., Pierreux, C.E., Odom, D.T., Peers, B., Lemaigre, F.P., 2012. A feedback loop between the liver-enriched transcription factor network and miR-122 controls hepatocyte differentiation. *Gastroenterology* 142, 119-129.
- Laumonnerie, C., Tong, Y.G., Alstermark, H., Wilson, S.I., 2015. Commissural axonal corridors instruct neuronal migration in the mouse spinal cord. *Nat Commun* 6, 7028.
- Lee, S., Lee, B., Joshi, K., Pfaff, S.L., Lee, J.W., Lee, S.K., 2008. A regulatory network to segregate the identity of neuronal subtypes. *Dev Cell* 14, 877-889.
- Lee, S., Lee, B., Lee, J.W., Lee, S.K., 2009. Retinoid signaling and neurogenin2 function are coupled for the specification of spinal motor neurons through a chromatin modifier CBP. *Neuron* 62, 641-654.
- Lee, S.K., Pfaff, S.L., 2001. Transcriptional networks regulating neuronal identity in the developing spinal cord. *Nat Neurosci* 4 Suppl, 1183-1191.
- Lemaigre, F.P., Durviaux, S.M., Truong, O., Lannoy, V.J., Hsuan, J.J., Rousseau, G.G., 1996. Hepatocyte nuclear factor 6, a transcription factor that contains a novel type of homeodomain and a single cut domain. *Proc Natl Acad Sci U S A* 93, 9460-9464.
- Lemon, R.N., 2008. Descending pathways in motor control. *Annu Rev Neurosci* 31, 195-218.
- Li, S., Weidenfeld, J., Morrissey, E.E., 2004. Transcriptional and DNA binding activity of the Foxp1/2/4 family is modulated by heterotypic and homotypic protein interactions. *Molecular and cellular biology* 24, 809-822.
- Li, Y., Cong, B., Ma, C., Qi, Q., Fu, L., Zhang, G., Min, Z., 2011. Expression of Nurr1 during rat brain and spinal cord development. *Neurosci Lett* 488, 49-54.
- Lillicrop, K.A., Latchman, D.S., 1992. Alternative splicing of the Oct-2 transcription factor RNA is differentially regulated in neuronal cells and B cells and results in protein isoforms with opposite effects on the activity of octamer/TAATGARAT-containing promoters. *J Biol Chem* 267, 24960-24965.

- Lin, J.H., Saito, T., Anderson, D.J., Lance-Jones, C., Jessell, T.M., Arber, S., 1998. Functionally related motor neuron pool and muscle sensory afferent subtypes defined by coordinate ETS gene expression. *Cell* 95, 393-407.
- Litingtung, Y., Chiang, C., 2000. Specification of ventral neuron types is mediated by an antagonistic interaction between Shh and Gli3. *Nature neuroscience* 3, 979-985.
- Liu, B., Liu, Z., Chen, T., Li, H., Qiang, B., Yuan, J., Peng, X., Qiu, M., 2007. Selective expression of Bhlhb5 in subsets of early-born interneurons and late-born association neurons in the spinal cord. *Dev Dyn* 236, 829-835.
- Liu, J.P., 2006. The function of growth/differentiation factor 11 (Gdf11) in rostrocaudal patterning of the developing spinal cord. *Development (Cambridge, England)* 133, 2865-2874.
- Liu, J.P., Laufer, E., Jessell, T.M., 2001. Assigning the positional identity of spinal motor neurons: rostrocaudal patterning of Hox-c expression by FGFs, Gdf11, and retinoids. *Neuron* 32, 997-1012.
- Liu, Y.Z., Lillycrop, K.A., Latchman, D.S., 1995. Regulated splicing of the Oct-2 transcription factor RNA in neuronal cells. *Neurosci Lett* 183, 8-12.
- Liu, Z., Hu, X., Huang, C., Zheng, K., Takebayashi, H., Cao, C., Qiu, M., 2014. Olig3 is not involved in the ventral patterning of spinal cord. *PLoS One* 9, e111076.
- Lu, D.C., Niu, T., Alaynick, W.A., 2015. Molecular and cellular development of spinal cord locomotor circuitry. *Front Mol Neurosci* 8, 25.
- Lundfald, L., Restrepo, C.E., Butt, S.J., Peng, C.Y., Droho, S., Endo, T., Zeilhofer, H.U., Sharma, K., Kiehn, O., 2007. Phenotype of V2-derived interneurons and their relationship to the axon guidance molecule EphA4 in the developing mouse spinal cord. *Eur J Neurosci* 26, 2989-3002.
- Lupo, G., Harris, W.A., Lewis, K.E., 2006. Mechanisms of ventral patterning in the vertebrate nervous system. *Nat Rev Neurosci* 7, 103-114.
- Luxenhofer, G., Helmbrecht, M.S., Langhoff, J., Giusti, S.A., Refojo, D., Huber, A.B., 2014. MicroRNA-9 promotes the switch from early-born to late-born motor neuron populations by regulating Onecut transcription factor expression. *Dev Biol* 386, 358-370.

- Maden, M., 2002. Retinoid signalling in the development of the central nervous system. *Nat Rev Neurosci* 3, 843-853.
- Maier, E.A., Dusing, M.R., Wiginton, D.A., 2006. Temporal regulation of enhancer function in intestinal epithelium: a role for Onecut factors. *J Biol Chem* 281, 32263-32271.
- Margagliotti, S., Clotman, F., Pierreux, C.E., Beaudry, J.B., Jacquemin, P., Rousseau, G.G., Lemaigre, F.P., 2007. The Onecut transcription factors HNF-6/OC-1 and OC-2 regulate early liver expansion by controlling hepatoblast migration. *Dev Biol* 311, 579-589.
- Marthiens, V., Gavard, J., Padilla, F., Monnet, C., Castellani, V., Lambert, M., Mege, R.M., 2005. A novel function for cadherin-11 in the regulation of motor axon elongation and fasciculation. *Mol Cell Neurosci* 28, 715-726.
- Marthiens, V., Padilla, F., Lambert, M., Mege, R.M., 2002. Complementary expression and regulation of cadherins 6 and 11 during specific steps of motoneuron differentiation. *Mol Cell Neurosci* 20, 458-475.
- Marti, E., Bovolenta, P., 2002. Sonic hedgehog in CNS development: one signal, multiple outputs. *Trends Neurosci* 25, 89-96.
- Matise, M.P., Joyner, A.L., 1997. Expression patterns of developmental control genes in normal and *Engrailed-1* mutant mouse spinal cord reveal early diversity in developing interneurons. *J Neurosci* 17, 7805-7816.
- Matthews, R.P., Lorent, K., Pack, M., 2008. Transcription factor onecut3 regulates intrahepatic biliary development in zebrafish. *Dev Dyn* 237, 124-131.
- McDonagh, J.C., Gorman, R.B., Gilliam, E.E., Hornby, T.G., Reinking, R.M., Stuart, D.G., 1998. Properties of spinal motoneurons and interneurons in the adult turtle: provisional classification by cluster analysis. *J Comp Neurol* 400, 544-570.
- McDonagh, J.C., Gorman, R.B., Gilliam, E.E., Hornby, T.G., Reinking, R.M., Stuart, D.G., 1999. Electrophysiological and morphological properties of neurons in the ventral horn of the turtle spinal cord. *Journal of physiology, Paris* 93, 3-16.
- Misra, K., Luo, H., Li, S., Matise, M., Xiang, M., 2014. Asymmetric activation of Dll4-Notch signaling by *Foxn4* and proneural factors activates BMP/TGF $\beta$  signaling to specify V2b interneurons in the spinal cord. *Development* 141, 187-198.

- Moens, C.B., Selleri, L., 2006. Hox cofactors in vertebrate development. *Developmental biology* 291, 193-206.
- Moran-Rivard, L., Kagawa, T., Saueressig, H., Gross, M.K., Burrill, J., Goulding, M., 2001. Evx1 is a postmitotic determinant of v0 interneuron identity in the spinal cord. *Neuron* 29, 385-399.
- Morikawa, Y., Hisaoka, T., Senba, E., 2009a. Characterization of Foxp2-expressing cells in the developing spinal cord. *Neuroscience* 162, 1150-1162.
- Morikawa, Y., Komori, T., Hisaoka, T., Senba, E., 2009b. Detailed expression pattern of Foxp1 and its possible roles in neurons of the spinal cord during embryogenesis. *Dev Neurosci* 31, 511-522.
- 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 & development* 19, 733-743.
- Muller, T., Brohmann, H., Pierani, A., Heppenstall, P.A., Lewin, G.R., Jessell, T.M., Birchmeier, C., 2002. The homeodomain factor Ibx1 distinguishes two major programs of neuronal differentiation in the dorsal spinal cord. *Neuron* 34, 551-562.
- Muroyama, Y., Fujiwara, Y., Orkin, S.H., Rowitch, D.H., 2005. Specification of astrocytes by bHLH protein SCL in a restricted region of the neural tube. *Nature* 438, 360-363.
- Nguyen, D.N., Rohrbaugh, M., Lai, Z., 2000. The Drosophila homolog of Onecut homeodomain proteins is a neural-specific transcriptional activator with a potential role in regulating neural differentiation. *Mech Dev* 97, 57-72.
- Nordstrom, U., Maier, E., Jessell, T.M., Edlund, T., 2006. An early role for WNT signaling in specifying neural patterns of Cdx and Hox gene expression and motor neuron subtype identity. *PLoS Biol* 4, e252.
- Novitch, B.G., Wichterle, H., Jessell, T.M., Sockanathan, S., 2003. A requirement for retinoic acid-mediated transcriptional activation in ventral neural patterning and motor neuron specification. *Neuron* 40, 81-95.

- Ono, Y., Nakatani, T., Sakamoto, Y., Mizuhara, E., Minaki, Y., Kumai, M., Hamaguchi, A., Nishimura, M., Inoue, Y., Hayashi, H., Takahashi, J., Imai, T., 2007. Differences in neurogenic potential in floor plate cells along an anteroposterior location: midbrain dopaminergic neurons originate from mesencephalic floor plate cells. *Development (Cambridge, England)* 134, 3213-3225.
- Osakada, F., Mori, T., Cetin, A.H., Marshel, J.H., Virgen, B., Callaway, E.M., 2011. New rabies virus variants for monitoring and manipulating activity and gene expression in defined neural circuits. *Neuron* 71, 617-631.
- Otim, O., Amore, G., Minokawa, T., McClay, D.R., Davidson, E.H., 2004. SpHnf6, a transcription factor that executes multiple functions in sea urchin embryogenesis. *Dev Biol* 273, 226-243.
- Palmesino, E., Rousso, D.L., Kao, T.J., Klar, A., Laufer, E., Uemura, O., Okamoto, H., Novitsch, B.G., Kania, A., 2010. Foxp1 and Ihx1 coordinate motor neuron migration with axon trajectory choice by gating Reelin signalling. *PLoS Biol* 8, e1000446.
- Panayi, H., Panayiotou, E., Orford, M., Genethliou, N., Mean, R., Lapathitis, G., Li, S., Xiang, M., Kessaris, N., Richardson, W.D., Malas, S., 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.
- Parras, C.M., Schuurmans, C., Scardigli, R., Kim, J., Anderson, D.J., Guillemot, F., 2002. Divergent functions of the proneural genes Mash1 and Ngn2 in the specification of neuronal subtype identity. *Genes & development* 16, 324-338.
- Pattyn, A., Vallstedt, A., Dias, J.M., Sander, M., Ericson, J., 2003. Complementary roles for Nkx6 and Nkx2 class proteins in the establishment of motoneuron identity in the hindbrain. *Development* 130, 4149-4159.
- 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.
- Peng, C.Y., Yajima, H., Burns, C.E., Zon, L.I., Sisodia, S.S., Pfaff, S.L., Sharma, K., 2007. Notch and MAML signaling drives Scl-dependent interneuron diversity in the spinal cord. *Neuron* 53, 813-827.

Pfisterer, P., Annweiler, A., Ullmer, C., Corcoran, L.M., Wirth, T., 1994. Differential transactivation potential of Oct1 and Oct2 is determined by additional B cell-specific activities. *EMBO J* 13, 1654-1663.

Phelps, P.E., Rich, R., Dupuy-Davies, S., Rios, Y., Wong, T., 2002. Evidence for a cell-specific action of Reelin in the spinal cord. *Dev Biol* 244, 180-198.

Philippidou, P., Dasen, J.S., 2013. Hox genes: choreographers in neural development, architects of circuit organization. *Neuron* 80, 12-34.

Pierani, A., Brenner-Morton, S., Chiang, C., Jessell, T.M., 1999. A sonic hedgehog-independent, retinoid-activated pathway of neurogenesis in the ventral spinal cord. *Cell* 97, 903-915.

Pierani, A., Moran-Rivard, L., Sunshine, M.J., Littman, D.R., Goulding, M., Jessell, T.M., 2001. Control of interneuron fate in the developing spinal cord by the progenitor homeodomain protein Dbx1. *Neuron* 29, 367-384.

Pierreux, C.E., Poll, A.V., Kemp, C.R., Clotman, F., Maestro, M.A., Cordi, S., Ferrer, J., Leyns, L., Rousseau, G.G., Lemaigre, F.P., 2006. The transcription factor hepatocyte nuclear factor-6 controls the development of pancreatic ducts in the mouse. *Gastroenterology* 130, 532-541.

Pierreux, C.E., Vanhorenbeeck, V., Jacquemin, P., Lemaigre, F.P., Rousseau, G.G., 2004. The transcription factor hepatocyte nuclear factor-6/Onecut-1 controls the expression of its paralog Onecut-3 in developing mouse endoderm. *J Biol Chem* 279, 51298-51304.

Pivetta, C., Esposito, M.S., Sigrist, M., Arber, S., 2014. Motor-circuit communication matrix from spinal cord to brainstem neurons revealed by developmental origin. *Cell* 156, 537-548.

Plumb-Rudewiez, N., Clotman, F., Strick-Marchand, H., Pierreux, C.E., Weiss, M.C., Rousseau, G.G., Lemaigre, F.P., 2004. Transcription factor HNF-6/OC-1 inhibits the stimulation of the HNF-3alpha/Foxa1 gene by TGF-beta in mouse liver. *Hepatology* 40, 1266-1274.

Poirier, K., Van Esch, H., Friocourt, G., Saillour, Y., Bahi, N., Backer, S., Souil, E., Castelnau-Ptakhine, L., Beldjord, C., Francis, F., Bienvenu, T., Chelly, J., 2004. Neuroanatomical distribution of ARX in brain and its localisation in GABAergic neurons. *Brain Res Mol Brain Res* 122, 35-46.

Poustka, A.J., Kuhn, A., Radosavljevic, V., Wellenreuther, R., Lehrach, H., Panopoulou, G., 2004. On the origin of the chordate central nervous system: expression of onecut in the sea urchin embryo. *Evol Dev* 6, 227-236.

Prasad, T., Wang, X., Gray, P.A., Weiner, J.A., 2008. A differential developmental pattern of spinal interneuron apoptosis during synaptogenesis: insights from genetic analyses of the protocadherin-gamma gene cluster. *Development* (Cambridge, England) 135, 4153-4164.

Qiu, M., Shimamura, K., Sussel, L., Chen, S., Rubenstein, J.L., 1998. Control of anteroposterior and dorsoventral domains of Nkx-6.1 gene expression relative to other Nkx genes during vertebrate CNS development. *Mech Dev* 72, 77-88.

Radhakrishnan, B., Alwin Prem Anand, A., 2016. Role of miRNA-9 in Brain Development. *J Exp Neurosci* 10, 101-120.

Ramirez-Jarquin, U.N., Tapia, R., 2018. Excitatory and Inhibitory Neuronal Circuits in the Spinal Cord and Their Role in the Control of Motor Neuron Function and Degeneration. *ACS Chem Neurosci* 9, 211-216.

Rausa, F., Samadani, U., Ye, H., Lim, L., Fletcher, C.F., Jenkins, N.A., Copeland, N.G., Costa, R.H., 1997. The cut-homeodomain transcriptional activator HNF-6 is coexpressed with its target gene HNF-3 beta in the developing murine liver and pancreas. *Dev Biol* 192, 228-246.

Rausa, F.M., 3rd, Hughes, D.E., Costa, R.H., 2004. Stability of the hepatocyte nuclear factor 6 transcription factor requires acetylation by the CREB-binding protein coactivator. *J Biol Chem* 279, 43070-43076.

Renoncourt, Y., Carroll, P., Filippi, P., Arce, V., Alonso, S., 1998. Neurons derived in vitro from ES cells express homeoproteins characteristic of motoneurons and interneurons. *Mechanisms of development* 79, 185-197.

Renshaw, B., 1946. Central effects of centripetal impulses in axons of spinal ventral roots. *J Neurophysiol* 9, 191-204.

Roberts, V.J., van Dijk, M.A., Murre, C., 1995. Localization of Pbx1 transcripts in developing rat embryos. *Mechanisms of development* 51, 193-198.



## Bibliography

---

Ross, S.E., Mardinly, A.R., McCord, A.E., Zurawski, J., Cohen, S., Jung, C., Hu, L., Mok, S.I., Shah, A., Savner, E.M., Tolias, C., Corfas, R., Chen, S., Inquimbert, P., Xu, Y., McInnes, R.R., Rice, F.L., Corfas, G., Ma, Q., Woolf, C.J., Greenberg, M.E., 2010. Loss of inhibitory interneurons in the dorsal spinal cord and elevated itch in *Bhlhb5* mutant mice. *Neuron* 65, 886-898.

Ross, S.E., McCord, A.E., Jung, C., Atan, D., Mok, S.I., Hemberg, M., Kim, T.K., Salogiannis, J., Hu, L., Cohen, S., Lin, Y., Harrar, D., McInnes, R.R., Greenberg, M.E., 2012. *Bhlhb5* and *Prdm8* form a repressor complex involved in neuronal circuit assembly. *Neuron* 73, 292-303.

Rousso, D.L., Gaber, Z.B., Wellik, D., Morrissey, E.E., Novitch, B.G., 2008. Coordinated actions of the forkhead protein *Foxp1* and *Hox* proteins in the columnar organization of spinal motor neurons. *Neuron* 59, 226-240.

Rousso, D.L., Pearson, C.A., Gaber, Z.B., Miquelajauregui, A., Li, S., Portera-Cailliau, C., Morrissey, E.E., Novitch, B.G., 2012. *Foxp*-mediated suppression of *N-cadherin* regulates neuroepithelial character and progenitor maintenance in the CNS. *Neuron* 74, 314-330.

Roy, A., Francius, C., Rousso, D.L., Seuntjens, E., Debruyne, J., Luxenhofer, G., Huber, A.B., Huylebroeck, D., Novitch, B.G., Clotman, F., 2012. *Onecut* transcription factors act upstream of *Isl1* to regulate spinal motoneuron diversification. *Development* 139, 3109-3119.

Ryall, R.W., Piercey, M.F., Polosa, C., 1971. Intersegmental and intrasegmental distribution of mutual inhibition of Renshaw cells. *J Neurophysiol* 34, 700-707.

Samadani, U., Costa, R.H., 1996. The transcriptional activator hepatocyte nuclear factor 6 regulates liver gene expression. *Mol Cell Biol* 16, 6273-6284.

Sander, M., Paydar, S., Ericson, J., Briscoe, J., Berber, E., German, M., Jessell, T.M., Rubenstein, J.L., 2000. Ventral neural patterning by *Nkx* homeobox genes: *Nkx6.1* controls somatic motor neuron and ventral interneuron fates. *Genes Dev* 14, 2134-2139.

Sapir, T., Geiman, E.J., Wang, Z., Velasquez, T., Mitsui, S., Yoshihara, Y., Frank, E., Alvarez, F.J., Goulding, M., 2004. *Pax6* and *engrailed 1* regulate two distinct aspects of rensaw cell development. *J Neurosci* 24, 1255-1264.

- Sapkota, D., Chintala, H., Wu, F., Fliesler, S.J., Hu, Z., Mu, X., 2014. Onecut1 and Onecut2 redundantly regulate early retinal cell fates during development. *Proc Natl Acad Sci U S A* 111, E4086-4095.
- Sasakura, Y., Makabe, K.W., 2001. A gene encoding a new ONECUT class homeodomain protein in the ascidian *Halocynthia roretzi* functions in the differentiation and specification of neural cells in ascidian embryogenesis. *Mech Dev* 104, 37-48.
- Saueressig, H., Burrill, J., Goulding, M., 1999. Engrailed-1 and netrin-1 regulate axon pathfinding by association interneurons that project to motor neurons. *Development* 126, 4201-4212.
- Schotland, J.L., Tresch, M.C., 1997. Segmental and propriospinal projection systems of frog lumbar interneurons. *Exp Brain Res* 116, 283-298.
- Schreiber, E., Harshman, K., Kemler, I., Malipiero, U., Schaffner, W., Fontana, A., 1990. Astrocytes and glioblastoma cells express novel octamer-DNA binding proteins distinct from the ubiquitous Oct-1 and B cell type Oct-2 proteins. *Nucleic Acids Res* 18, 5495-5503.
- Siembab, V.C., Smith, C.A., Zagoraïou, L., Berrocal, M.C., Mentis, G.Z., Alvarez, F.J., 2010. Target selection of proprioceptive and motor axon synapses on neonatal V1-derived Ia inhibitory interneurons and Renshaw cells. *J Comp Neurol* 518, 4675-4701.
- Simion, A., Laudadio, I., Prevot, P.P., Raynaud, P., Lemaigre, F.P., Jacquemin, P., 2010. MiR-495 and miR-218 regulate the expression of the Onecut transcription factors HNF-6 and OC-2. *Biochem Biophys Res Commun* 391, 293-298.
- Skaggs, K., Martin, D.M., Novitch, B.G., 2011. Regulation of spinal interneuron development by the Olig-related protein Bhlhb5 and Notch signaling. *Development* 138, 3199-3211.
- Smith, E., Hargrave, M., Yamada, T., Begley, C.G., Little, M.H., 2002. Coexpression of SCL and GATA3 in the V2 interneurons of the developing mouse spinal cord. *Dev Dyn* 224, 231-237.
- Sockanathan, S., Jessell, T.M., 1998. Motor neuron-derived retinoid signaling specifies the subtype identity of spinal motor neurons. *Cell* 94, 503-514.

- Sockanathan, S., Perlmann, T., Jessell, T.M., 2003. Retinoid receptor signaling in postmitotic motor neurons regulates rostrocaudal positional identity and axonal projection pattern. *Neuron* 40, 97-111.
- Southwood, C., He, C., Garbern, J., Kamholz, J., Arroyo, E., Gow, A., 2004. CNS myelin paranodes require Nkx6-2 homeoprotein transcriptional activity for normal structure. *J Neurosci* 24, 11215-11225.
- Stam, F.J., Hendricks, T.J., Zhang, J., Geiman, E.J., Francius, C., Labosky, P.A., Clotman, F., Goulding, M., 2012. Renshaw cell interneuron specialization is controlled by a temporally restricted transcription factor program. *Development* 139, 179-190.
- Stern, S., Tanaka, M., Herr, W., 1989. The Oct-1 homoeodomain directs formation of a multiprotein-DNA complex with the HSV transactivator VP16. *Nature* 341, 624-630.
- Stoykova, A.S., Sterrer, S., Erselius, J.R., Hatzopoulos, A.K., Gruss, P., 1992. Mini-Oct and Oct-2c: two novel, functionally diverse murine Oct-2 gene products are differentially expressed in the CNS. *Neuron* 8, 541-558.
- Sturm, R.A., Herr, W., 1988. The POU domain is a bipartite DNA-binding structure. *Nature* 336, 601-604.
- Surmeli, G., Akay, T., Ippolito, G.C., Tucker, P.W., Jessell, T.M., 2011. Patterns of spinal sensory-motor connectivity prescribed by a dorsoventral positional template. *Cell* 147, 653-665.
- Sweeney, L.B., Bikoff, J.B., Gabitto, M.I., Brenner-Morton, S., Baek, M., Yang, J.H., Tabak, E.G., Dasen, J.S., Kintner, C.R., Jessell, T.M., 2018. Origin and Segmental Diversity of Spinal Inhibitory Interneurons. *Neuron* 97, 341-355 e343.
- Takahashi, K., Liu, F.C., Hirokawa, K., Takahashi, H., 2008. Expression of Foxp4 in the developing and adult rat forebrain. *J Neurosci Res* 86, 3106-3116.
- Takebayashi, H., Ohtsuki, T., Uchida, T., Kawamoto, S., Okubo, K., Ikenaka, K., Takeichi, M., Chisaka, O., Nabeshima, Y., 2002. Non-overlapping expression of Olig3 and Olig2 in the embryonic neural tube. *Mechanisms of development* 113, 169-174.

- Talpalar, A.E., Bouvier, J., Borgius, L., Fortin, G., Pierani, A., Kiehn, O., 2013. Dual-mode operation of neuronal networks involved in left-right alternation. *Nature* 500, 85-88.
- Tam, W.Y., Leung, C.K., Tong, K.K., Kwan, K.M., 2011. Foxp4 is essential in maintenance of Purkinje cell dendritic arborization in the mouse cerebellum. *Neuroscience* 172, 562-571.
- Tamura, S., Morikawa, Y., Iwanishi, H., Hisaoka, T., Senba, E., 2003. Expression pattern of the winged-helix/forkhead transcription factor Foxp1 in the developing central nervous system. *Gene Expr Patterns* 3, 193-197.
- Tanaka, Y., Naruse, I., Hongo, T., Xu, M., Nakahata, T., Maekawa, T., Ishii, S., 2000. Extensive brain hemorrhage and embryonic lethality in a mouse null mutant of CREB-binding protein. *Mech Dev* 95, 133-145.
- Thaler, J.P., Lee, S.K., Jurata, L.W., Gill, G.N., Pfaff, S.L., 2002. LIM factor Lhx3 contributes to the specification of motor neuron and interneuron identity through cell-type-specific protein-protein interactions. *Cell* 110, 237-249.
- Thelie, A., Desiderio, S., Hanotel, J., Quigley, I., Van Driessche, B., Rodari, A., Borromeo, M.D., Kricha, S., Lahaye, F., Croce, J., Cerda-Moya, G., Ordonez Fernandez, J., Bolle, B., Lewis, K.E., Sander, M., Pierani, A., Schubert, M., Johnson, J.E., Kintner, C.R., Pieler, T., Van Lint, C., Henningfeld, K.A., Bellefroid, E.J., Van Campenhout, C., 2015. Prdm12 specifies V1 interneurons through cross-repressive interactions with Dbx1 and Nkx6 genes in *Xenopus*. *Development* 142, 3416-3428.
- Theodorou, E., Dalember, G., Heffelfinger, C., White, E., Weissman, S., Corcoran, L., Snyder, M., 2009. A high throughput embryonic stem cell screen identifies Oct-2 as a bifunctional regulator of neuronal differentiation. *Genes Dev* 23, 575-588.
- Todd, K.J., Lan-Chow-Wing, N., Salin-Cantegrel, A., Cotter, A., Zagami, C.J., Lo, R., Stifani, S., 2012. Establishment of motor neuron-V3 interneuron progenitor domain boundary in ventral spinal cord requires Groucho-mediated transcriptional corepression. *PLoS One* 7, e31176.
- Tripodi, M., Stepien, A.E., Arber, S., 2011. Motor antagonism exposed by spatial segregation and timing of neurogenesis. *Nature* 479, 61-66.

- Tsuchida, T., Ensini, M., Morton, S.B., Baldassare, M., Edlund, T., Jessell, T.M., Pfaff, S.L., 1994. Topographic organization of embryonic motor neurons defined by expression of LIM homeobox genes. *Cell* 79, 957-970.
- Uemura, O., Okada, Y., Ando, H., Guedj, M., Higashijima, S., Shimazaki, T., Chino, N., Okano, H., Okamoto, H., 2005. Comparative functional genomics revealed conservation and diversification of three enhancers of the *isl1* gene for motor and sensory neuron-specific expression. *Dev Biol* 278, 587-606.
- Ulloa, F., Briscoe, J., 2007. Morphogens and the control of cell proliferation and patterning in the spinal cord. *Cell Cycle* 6, 2640-2649.
- Vallstedt, A., Muhr, J., Pattyn, A., Pierani, A., Mendelsohn, M., Sander, M., Jessell, T.M., Ericson, J., 2001. Different levels of repressor activity assign redundant and specific roles to *Nkx6* genes in motor neuron and interneuron specification. *Neuron* 31, 743-755.
- Vanhorenbeeck, V., Jacquemin, P., Lemaigre, F.P., Rousseau, G.G., 2002. OC-3, a novel mammalian member of the ONECUT class of transcription factors. *Biochem Biophys Res Commun* 292, 848-854.
- Vanhorenbeeck, V., Jenny, M., Cornut, J.F., Gradwohl, G., Lemaigre, F.P., Rousseau, G.G., Jacquemin, P., 2007. Role of the *Onecut* transcription factors in pancreas morphogenesis and in pancreatic and enteric endocrine differentiation. *Dev Biol* 305, 685-694.
- Vermot, J., Schuhbaur, B., Le Mouellic, H., McCaffery, P., Garnier, J.M., Hentsch, D., Brulet, P., Niederreither, K., Chambon, P., Dolle, P., Le Roux, I., 2005. Retinaldehyde dehydrogenase 2 and *Hoxc8* are required in the murine brachial spinal cord for the specification of *Lim1+* motoneurons and the correct distribution of *Islet1+* motoneurons. *Development (Cambridge, England)* 132, 1611-1621.
- Vernes, S.C., Oliver, P.L., Spiteri, E., Lockstone, H.E., Puliyadi, R., Taylor, J.M., Ho, J., Mombereau, C., Brewer, A., Lowy, E., Nicod, J., Groszer, M., Baban, D., Sahgal, N., Cazier, J.B., Ragoussis, J., Davies, K.E., Geschwind, D.H., Fisher, S.E., 2011. *Foxp2* regulates gene networks implicated in neurite outgrowth in the developing brain. *PLoS genetics* 7, e1002145.

## Bibliography

---

- Villeda, S.A., Akopians, A.L., Babayan, A.H., Basbaum, A.I., Phelps, P.E., 2006. Absence of Reelin results in altered nociception and aberrant neuronal positioning in the dorsal spinal cord. *Neuroscience* 139, 1385-1396.
- Wall, N.R., Wickersham, I.R., Cetin, A., De La Parra, M., Callaway, E.M., 2010. Monosynaptic circuit tracing in vivo through Cre-dependent targeting and complementation of modified rabies virus. *Proceedings of the National Academy of Sciences of the United States of America* 107, 21848-21853.
- Wende, H., Lechner, S.G., Cheret, C., Bourane, S., Kolanczyk, M.E., Pattyn, A., Reuter, K., Munier, F.L., Carroll, P., Lewin, G.R., Birchmeier, C., 2012. The transcription factor c-Maf controls touch receptor development and function. *Science (New York, N.Y)* 335, 1373-1376.
- Wilson, L., Gale, E., Chambers, D., Maden, M., 2004. Retinoic acid and the control of dorsoventral patterning in the avian spinal cord. *Developmental biology* 269, 433-446.
- Wirth, T., Priess, A., Annweiler, A., Zwilling, S., Oeler, B., 1991. Multiple Oct2 isoforms are generated by alternative splicing. *Nucleic Acids Res* 19, 43-51.
- Wood, J.N., Lillycrop, K.A., Dent, C.L., Ninkina, N.N., Beech, M.M., Willoughby, J.J., Winter, J., Latchman, D.S., 1992. Regulation of expression of the neuronal POU protein Oct-2 by nerve growth factor. *J Biol Chem* 267, 17787-17791.
- Wu, F., Sapkota, D., Li, R., Mu, X., 2012. Onecut 1 and Onecut 2 are potential regulators of mouse retinal development. *J Comp Neurol* 520, 952-969.
- Wu, Y., Wang, G., Scott, S.A., Capecchi, M.R., 2008. Hoxc10 and Hoxd10 regulate mouse columnar, divisional and motor pool identity of lumbar motoneurons. *Development (Cambridge, England)* 135, 171-182.
- Yip, J.W., Yip, Y.P., Nakajima, K., Capriotti, C., 2000. Reelin controls position of autonomic neurons in the spinal cord. *Proc Natl Acad Sci U S A* 97, 8612-8616.
- Zagoraïou, L., Akay, T., Martin, J.F., Brownstone, R.M., Jessell, T.M., Miles, G.B., 2009. A cluster of cholinergic premotor interneurons modulates mouse locomotor activity. *Neuron* 64, 645-662.
- Zannino, D.A., Downes, G.B., Sagerstrom, C.G., 2014. prdm12b specifies the p1 progenitor domain and reveals a role for V1 interneurons in swim movements. *Dev Biol* 390, 247-260.

## Bibliography

---

Zhang, H., Ables, E.T., Pope, C.F., Washington, M.K., Hipkens, S., Means, A.L., Path, G., Seufert, J., Costa, R.H., Leiter, A.B., Magnuson, M.A., Gannon, M., 2009. Multiple, temporal-specific roles for HNF6 in pancreatic endocrine and ductal differentiation. *Mech Dev* 126, 958-973.

Zhang, J., Lanuza, G.M., Britz, O., Wang, Z., Siembab, V.C., Zhang, Y., Velasquez, T., Alvarez, F.J., Frank, E., Goulding, M., 2014. V1 and v2b interneurons secure the alternating flexor-extensor motor activity mice require for limbed locomotion. *Neuron* 82, 138-150.

Zhang, Y., Narayan, S., Geiman, E., Lanuza, G.M., Velasquez, T., Shanks, B., Akay, T., Dyck, J., Pearson, K., Gosgnach, S., Fan, C.M., Goulding, M., 2008. V3 spinal neurons establish a robust and balanced locomotor rhythm during walking. *Neuron* 60, 84-96.

Zhong, G., Droho, S., Crone, S.A., Dietz, S., Kwan, A.C., Webb, W.W., Sharma, K., Harris-Warrick, R.M., 2010. Electrophysiological characterization of V2a interneurons and their locomotor-related activity in the neonatal mouse spinal cord. *J Neurosci* 30, 170-182.

Zhou, Y., Yamamoto, M., Engel, J.D., 2000. GATA2 is required for the generation of V2 interneurons. *Development (Cambridge, England)* 127, 3829-3838.

Ziskind-Conhaim, L., Hochman, S., 2017. Diversity of molecularly defined spinal interneurons engaged in mammalian locomotor pattern generation. *J Neurophysiol* 118, 2956-2974.