The FEBS Journal

FEBS PRESS science publishing by scientists

REVIEW

Insights into the mechanisms of neuron generation and specification in the zebrafish ventral spinal cord

Gokhan Cucun, Melina Köhler, Sabrina Pfitsch and Sepand Rastegar

Institute for Biological and Chemical Systems – Biological Information Processing (IBCS-BIP), Karlsruhe Institute of Technology (KIT), Eggenstein-Leopoldshafen, Germany

Keywords
central nervous system; lateral floor plate; neurons; patterning; progenitor domain; specification; spinal cord; V2 domain; zebrafish

Correspondence
S. Rastegar, Institute for Biological and Chemical Systems – Biological Information Processing (IBCS-BIP), Karlsruhe Institute of Technology (KIT), Campus Nord, Hermann-von-Helmholtz-Platz 1, 76344 Eggenstein-Leopoldshafen, Germany
Tel: +49 721 608 22507
E-mail: sepand.rastegar@kit.edu

Gokhan Cucun and Melina Köhler contributed equally to this article

(Received 2 May 2023, revised 20 June 2023, accepted 25 July 2023)

doi:10.1111/febs.16913

The vertebrate nervous system is composed of a wide range of neurons and complex synaptic connections, raising the intriguing question of how neuronal diversity is generated. The spinal cord provides an excellent model for exploring the mechanisms governing neuronal diversity due to its simple neural network and the conserved molecular processes involved in neuron formation and specification during evolution. This review specifically examines two distinct progenitor domains present in the zebrafish ventral spinal cord: the lateral floor plate (LFP) and the p2 progenitor domain. The LFP is responsible for the production of GABAergic Kolmer–Agduhr neurons (KA″), glutamatergic V3 neurons, and intraspinal serotonergic neurons, while the p2 domain generates V2 precursors that subsequently differentiate into three unique subpopulations of V2 neurons, namely glutamatergic V2a, GABAergic V2b, and glycinergic V2s. Based on recent findings, we will examine the fundamental signaling pathways and transcription factors that play a key role in the specification of these diverse neurons and neuronal subtypes derived from the LFP and p2 progenitor domains.

Introduction

The spinal cord is an excellent model for studying the mechanisms that regulate the generation of neuronal diversity because of its simple neural network and the conserved molecular mechanisms involved in neuron formation and specification. In the vertebrate spinal cord, neurons and glia are organized into distinct classes that arise from separate progenitor domains along the dorsoventral axis of the embryonic neural tube (Fig. 1A) [1–3]. The generation of different types of postmitotic neurons at different positions along the spinal cord axis is facilitated mainly by two important gradients. First, a dorsal to ventral gradient of morphogens, such as bone morphogenetic proteins (BMPs) and wingless-integrated (Wnt) released from the roof plate. Second, a ventral to dorsal gradient of Sonic Hedgehog (Shh) released from the floor plate. These gradients trigger the combinatorial expression of transcription factors (TFs) in the progenitor domains, leading to the production of distinct

Abbreviations
BMPs, bone morphogenetic proteins; CSF-cNs, cerebrospinal fluid-contacting neurons; dpf, days postfertilization; GABA, γ-aminobutyric acid; Gad, glutamic acid decarboxylase; hpf, hours post-fertilization; ISNs, intraspinal serotonergic neurons; KA, Kolmer–Agduhr; LFP, lateral floor plate; MFP, medial floor plate; MN, motor neuron; RP, roof plate; scRNA-seq, single-cell RNA sequencing; Shh, Sonic Hedgehog; TF, transcription factor; Wnt, wingless-integrated.

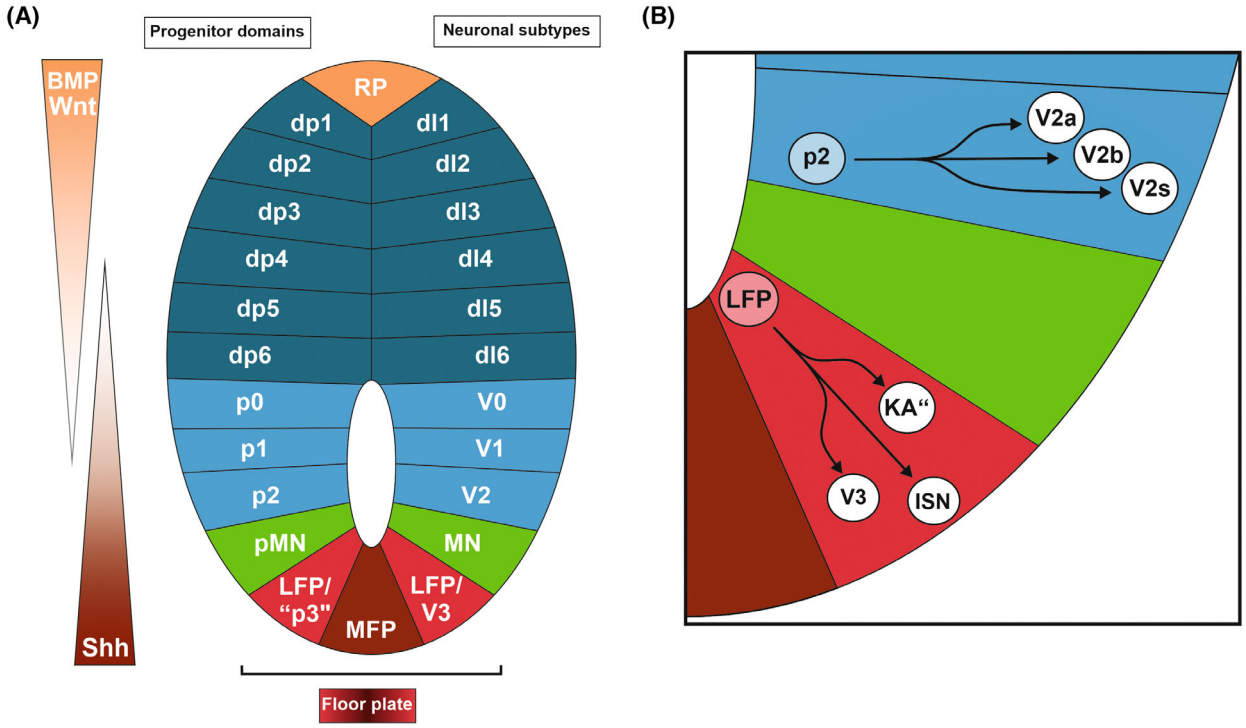

Fig. 1. Antagonistic actions of Shh and BMP/Wnt divide the spinal cord into distinct progenitor domains from which specific postmitotic neurons are generated. (A) The spinal cord is divided into 11 progenitor domains through the opposing actions of Shh, secreted from the MFP, and BMP/Wnt, released from the roof plate (RP). These domains generate distinct postmitotic neurons. (B) The LFP progenitor cells produce three neuronal populations—KA″, V3, and ISNs, while the progenitors in the p2 domain generate three V2 interneuron subtypes designated as V2a, V2b, and V2s. The drawing depicts a zebrafish spinal cord.

neuronal types (Fig. 1A) [1–4]. Ventral progenitor domains, including p3, pMN, p2, p1, and p0, give rise to multiple types of postmitotic neurons with distinct anatomy, processes, connections, and functions. Furthermore, recent evidence suggests that some postmitotic neurons may further diversify into molecularly and functionally distinct subpopulations [1–5].

This review will focus specifically on two zebrafish progenitor domains: lateral floor plate (LFP), which is homologous to the mouse p3 domain [6], and the p2 progenitor domain. The LFP plays a crucial role in the generation of neurons in the developing zebrafish spinal cord. During embryonic development, it sequentially produces different types of neurons, including KA″ neurons, V3 neurons, and intraspinal serotonergic neurons (ISNs) (Fig. 1B) [7–13]. On the contrary, the p2 precursor domain is responsible for the formation of three distinct subpopulations of V2 neurons, each with unique functions, namely V2a, V2b, and V2s interneurons (V2c in mouse) (Fig. 1B).

Recent studies have led to a better understanding of the molecular mechanisms underlying the formation and specification of these progenitor domains and their derivatives [13–24]. In addition to the signaling pathways mentioned above, several studies have demonstrated the important role of the Notch signaling pathway in determining the fate of ventral spinal cord progenitor cells [7–9,11,13,14,16,25]. Specifically, in ventral spinal cord progenitor domains, the Notch signaling pathway maintains progenitor cells in an undifferentiated state [9]. This mechanism ensures that only a limited number of cells within a precursor domain adopt a specific neuronal fate. By preventing neighboring cells from differentiating into the same cell type and depleting the pool of progenitors, the Notch pathway contributes to the generation of diverse neuronal populations, including KA″ cells, V3 interneurons, and ISNs. In addition, the Notch signaling pathway also influences binary fate decisions, ensuring that daughter cells derived from a precursor cell assume distinct fates. This mechanism contributes to the generation of various subtypes of neurons, such as the V2 interneurons (e.g., V2a, V2b, and V2s) [14,16].

The aim of this review was to provide a comprehensive and up-to-date overview of the latest advances in our understanding of neuron formation and specification in the zebrafish ventral spinal cord, with a focus on the genetic cascades and signaling pathways involved.

The zebrafish lateral floor plate is the most ventrally positioned neural progenitor domain of the spinal cord

The floor plate, located in the ventral region of the spinal cord, consists of two parts: the inner cells known as medial floor plate cells (MFP) and the cells on either side called LFP (Fig. 2A) [26]. Despite being adjacent and sharing certain molecular markers, these structures have distinct functions and originate from different embryonic sources [27,28]. The MFP arises from embryonic shield cells that migrate into the neural tube [29–32]. Its primary role in neural tube and axon formation is achieved through the secretion of signaling molecules Shh and Netrin-1, respectively (Table 1) [33–35]. On the contrary, the LFP consists of ventral neuroectodermal cells [11,28], whose fate is induced by Shh signals released by the MFP and the notochord [27,28,36–39]. Extensive genetic and fate mapping studies have demonstrated that the zebrafish LFP represents the most ventral progenitor domain of the spinal cord, analogous to the mouse p3 domain [7,9,13]. Similar to the mouse p3 domain, LFP cells are induced by Shh and express characteristic p3 domain markers such as *nkx2.2a/b* and *nkx2.9* (Fig. 2B, Table 1) [12,27,40]. Additionally, the expression of these marker genes in LFP cells relies on the presence of Shh. Recent research has further revealed that the LFP gives rise to V3 interneurons, KA″ cells, and ISNs, as shown in Fig. 1B [7,8,11,13]. Moreover, the LFP also generates a population of glial cells known as motor exit point glia [41–43]. In the subsequent section, we will provide a brief overview of the characteristics and roles of the three neuronal populations originating from LFP progenitor cells, along with a discussion on the molecular mechanisms necessary for their development and specification.

Table 1. Comparison of the marker gene expression profile of LFP and MFP.

	foxa2	*ntn1a*	*Shh*	*nkx2.2a/b nkx2.9*
LFP	+	+	+ From 36 hpf [99]	+
MFP	+	+	+	–

Function and features of KA″ cells, V3 interneurons, and ISNs developed from the zebrafish LFP

KA″ cells

Kolmer–Agduhr neurons, a subgroup of cerebrospinal fluid-contacting neurons (CSF-cNs), were initially identified by Kolmer and Agduhr (KA). These neurons extend their apical projection into the cerebral ventricles and the

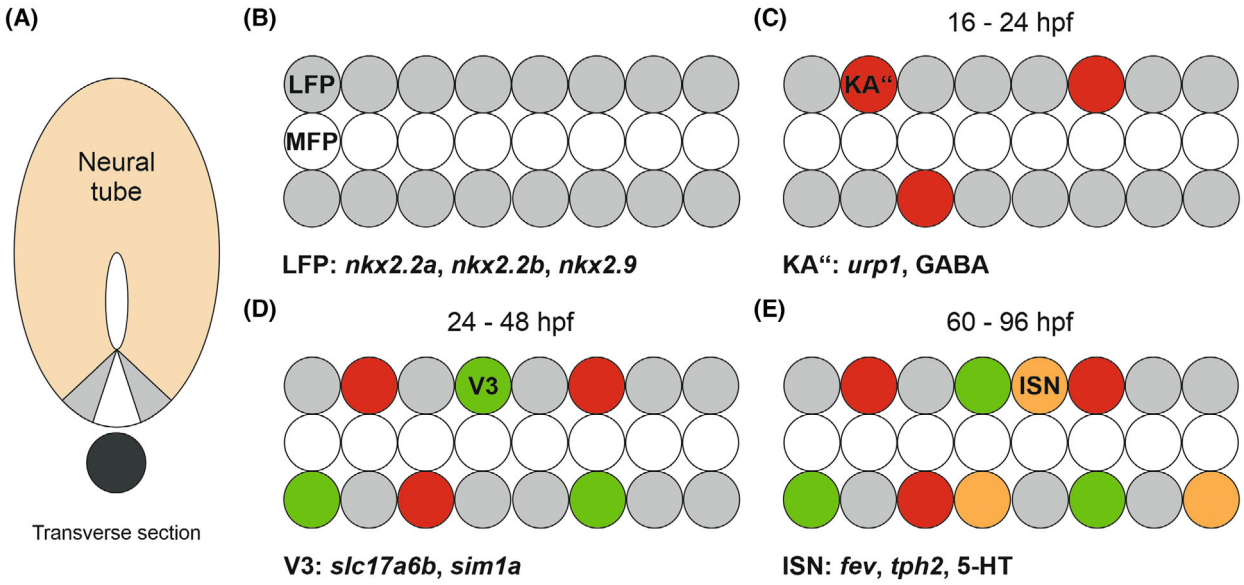

Fig. 2. Floor plate is organized into MFP and LFP. The LFP generates sequentially specific neuronal types that are characterized by distinct markers. (A) A transverse section through the zebrafish neural tube showing the ventral location of the MFP and LFP. (B–E) Images show the floor plate and the sequential specification of interneurons from the LFP (dorsal view). (B) LFP cells are positioned adjacent to MFP cells and are characterized by distinct markers. (C) GABAergic KA″ neurons arise early in development from LFP progenitor cells and express several specific markers. (D) Glutamatergic V3 neurons are generated between 24 and 48 hpf from the LFP progenitor. (E) Serotonergic neurons (5-HT), known as ISNs, are generated between 60 and 96 hpf from LFP progenitor cells. Adapted from Huang *et al.* [7] and Jacobs *et al.* [9].

central canal of the spinal cord [11,44,45]. They have a distinctive pear-shaped soma and express genes encoding glutamic acid decarboxylases (gads), exhibiting immunoreactivity to γ-aminobutyric acid (GABA) [46,47].

In zebrafish, two subtypes of KA cells have been identified, both in direct contact with the central canal and showing similar anatomical features. They are GABAergic and share the expression of various TFs such as *gata2a*, *gata3*, *tal1*, and *tal2* [12,45] (Fig. 2C). However, they originate from different progenitor domains [12,44,48,49].

KA′ neurons arise from the *olig2*-positive pMN domain around 10–12-h postfertilization (hpf) and are located dorsally compared with KA″ cells. Molecularly, KA′ neurons differ from KA″ neurons by specifically expressing the *somatostatin 1.1* gene (*sst1.1*) [44].

KA″ cells emerge from the LFP at approximately 16.5 hpf [7,9]. They occupy a ventral position in the spinal cord and, in addition to being GABAergic, transiently express *tph1a*, exhibiting temporary serotonergic characteristics [50,51]. Furthermore, they are distinct from KA′ cells due to their unique expression of *urotensin-related peptide 1* (*urp1*) and *urotensin II-related peptide 2* (*urp2*) [52].

As interneurons with processes in contact with the central canal, KA neurons are believed to detect changes and stimuli in the cerebrospinal fluid, possibly through the putative chemo- and/or mechanosensitive *polycystic kidney disease 2-like 1* channel (*pkd2l1*) [53,54]. They can transmit acquired information via projections to spinal neurons involved in locomotion, such as the primary commissural ascending interneurons and primary caudal motor neurons of the central pattern generators [44,55].

Consequently, it is hypothesized that mechanosensitive KA cells in zebrafish play a role in maintaining balance and body posture [44,56–58]. Additionally, they could serve as potential chemosensors, integrating cues from the cerebrospinal fluid and potentially possessing secretory functions themselves [44].

V3 interneurons

V3 cells are a distinct type of interneuron derived from the LFP and play a crucial role in locomotion regulation. Early identification of V3 interneurons is based on the expression of *sim1a*, while *slc17a6b* (formerly known as *vglut2a*) serves as a later marker gene (Fig. 2D) [9,11]. These excitatory glutamatergic interneurons, adjacent to KA″ cells in the LFP, are thought to be involved in the control of locomotor amplitude independently of locomotor frequency in zebrafish larvae [59,60]. Recent research has demonstrated that V3 cells are essential for recruiting spinal motor neurons and providing excitatory signals to activate them, ultimately leading to the initiation of fictive swimming in larval zebrafish [60].

Although not yet studied in zebrafish, work conducted on the mouse spinal cord have revealed the generation of different subpopulations of V3 interneurons at distinct developmental stages [61]. Later in this review, we will use the example of the zebrafish p2/V2 domain to illustrate the possible molecular mechanisms involved in generating the diversity of neuronal subpopulations.

Intraspinal serotonergic neurons

Recent single-cell RNA sequencing (scRNA-seq) approach revealed that ISNs are also derivatives of the LFP in zebrafish [13]. These late-born neurons are known to produce serotonin, along with the hindbrain raphe nuclei, and maintain detectable levels of this neurotransmitter in the spinal cord even after suprraspinal projections have been severed [62–65].

In zebrafish, ISNs with an oval-shaped morphology emerge around 2.5–3 days postfertilization (dpf) and undergo morphological changes until they mature at approximately 5 dpf (Fig. 2E) [10]. Their caudally extending processes appear to be axons terminating in enlarged growth cones, while their rostrally extending neurites branch into dendritic arbors [66,67]. Molecularly, ISNs can be identified by the expression of marker genes such as *fifth ewing variant* TF (*fev*; formerly known as *PC12 ETS factor 1*, *pet1*) (Fig. 2E) [68] and *tph2*, which encodes the rate-limiting enzyme Tph2 responsible for serotonin synthesis [69].

In contrast to ISNs in other vertebrates, zebrafish ISNs are abundant and evenly distributed along the anterior–posterior axis of the spinal cord [10,70–72]. This abundance suggests distinct functional properties compared with mammalian ISNs [10,70,71]. In zebrafish, ISNs play a crucial role in locomotion as they are closely positioned to motor neurons [66,67,72,73]. Interestingly, the development of ISNs and the establishment of beat-and-glide swimming patterns occur simultaneously at 4 dpf, and this capability is maintained throughout adulthood in zebrafish. Furthermore, ISNs contribute to the regeneration of spinal cord injuries and the restoration of motor circuitry by releasing serotonin, which promotes axonal regrowth of severed interneurons [74].

Molecular mechanisms of LFP specification

Several studies have shown that Shh, secreted by the MFP and the notochord, is essential for LFP

formation [27,40]. When Shh reaches its target cells, it triggers the production of active forms of zinc finger-containing Gli transcriptional effectors. Through mutant analysis of components in the Shh signaling pathway, researchers have confirmed the significance of these factors in inducing and forming the LFP [36–38]. Once active, Gli proteins bind to the regulatory sequences of downstream target genes of Shh, such as *nkx2.2a*, *nkx2.2b*, and *nkx2.9*, and they thereby induce the transcriptional expression of the respective genes in cells destined to become LFP precursor cells (Fig. 3) [75–77]. Loss-of-function studies on *nkx2* genes have further demonstrated that the activity of these TF genes is critical for the proper development of progenitor cells of the LFP [12].

Additionally, Schäfer *et al.* [11] demonstrated that the LFP is composed of heterogeneous cell populations in which p3, V3, and LFP progenitor cells are intermingled. Moreover, their mutant studies already suggested that the activity of Delta-Notch, Shh, and Nkx2.2 is involved in the generation of both LFP progenitor and p3 cells.

Recent studies shed light on the mechanisms underlying the specification of KA″ and V3 interneurons from LFP progenitor cells. In particular, the work of Huang and Jacobs using a photoconvertible reporter of signaling history has provided intriguing insights into the sequential and time-dependent manner of LFP progeny generation [7–9]. Especially, two of the major signaling pathways, Shh and Notch signaling, got into focus due to their involvement in governing cell fate specification and in maintaining progenitor fate in vertebrates, respectively [25]. Their work has elegantly shown that the two main signaling pathways are essential for the specification and maintenance of progenitor fate in the LFP [25].

Molecular mechanisms involved in KA″ cell, V3 interneuron, and ISN specification

KA″ cell specification

Cell lineage tracing experiments have provided insights into the development of KA″ cells from LFP progenitor cells. These progenitor cells express *nkx2.2a*, *nkx2.2b*, and *nkx2.9* and undergo asymmetric or symmetric cell divisions between approximately 16.5 and 24 hpf, giving rise to KA″ cells [7]. During KA″ specification, the *nkx2* family of TFs induces the expression of *gata2a*, which activates the expression of *gata3* and *tal2*. The expression of *tal2* is crucial for the transcription activation of *gad1b* (also known as *gad67*), which is involved in GABA synthesis (Fig. 3) [12,45].

In this model, LFP progenitor cells continue to be responsive to Shh signals, whereas differentiated KA″ cells lose their responsiveness over time. Interestingly, overexpression of Shh in KA″ cells does not restore their ability to respond to Shh signals. Additionally, once KA″ cells reach the postmitotic stage and complete their specification, they cannot revert back to the progenitor state even with extrinsic Shh signals [7]. The duration and termination of Shh signaling are critical for proper KA″ cell differentiation. Blocking

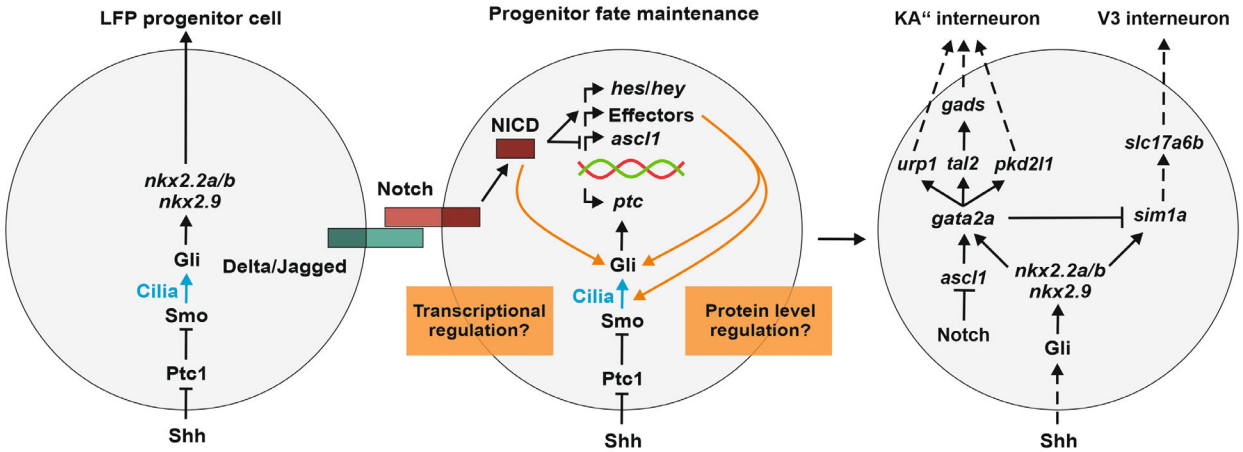

Fig. 3. Signaling pathways involved in the induction of LFP, the maintenance of LFP progenitor cells, and their differentiation into KA″ and V3 neurons are shown. (Left) Induction of LFP cells by the Shh pathway. (Middle) Both Shh and Notch signalling pathways are necessary to maintain LFP cells as progenitors. (Right) Inhibition of the Notch pathway allows the differentiation of LFP cells into KA″ (16–24 hpf) and V3 (24–48 hpf) neurons. During KA″ and V3 interneuron specification, crosstalk between the Shh and Notch signaling pathway could also be mediated via the regulation of transcription or protein levels as observed during central nervous system development.

Shh signaling at early stages leads to the elimination of KA″ cells, while blocking it later reduces their number partially. However, inhibiting the Shh pathway from 18 hpf onwards does not disrupt KA″ cell specification since differentiated cells no longer depend on active Shh signaling [7]. On the contrary, sustained Shh signaling caused by constitutively active upstream and downstream effectors results in an expansion of KA″ cells in the LFP and adjacent ventral domains of the neural tube. Interestingly, constitutive Shh signaling through forced *gli1* expression prevents signal termination and promotes the maintenance of the LFP progenitor state over KA″ cell specification [7,8]. Notch signaling, such as Shh signaling, is undetectable in KA″ cells, while LFP progenitors exhibit a high Notch response. The level and duration of Notch signaling play a crucial role in determining whether LFP progenitors are maintained or KA″ cells are specified (Fig. 3) [7,9]. The interplay between Shh and Notch signaling pathways regulates the transition from the progenitor state to differentiating cells. Notch effectors can maintain Shh responsiveness by facilitating the trafficking of important Shh receptors and regulating the transcription and protein levels of critical Gli activators. On the contrary, Gli factors have transcriptional control over components of the Notch signaling pathway. This intricate cross talk between Shh and Notch signaling pathways is crucial for the development of KA″ cells and may also regulate interneuron specification from the LFP [7,25].

In summary, Notch signaling is essential for Shh responsiveness, and both pathways are required to maintain the neural progenitor fate (Fig. 3). However, KA″ cell identity can only be established in the presence of Shh, and the final specification occurs through the attenuation of both pathways. Inhibiting Notch signaling allows the transition from the progenitor state to differentiation in KA″ cells (Fig. 3) [7].

V3 cell specification

V3 cell differentiation, such as KA″ cells, is initiated by *nkx2* TF members. These members induce the expression of the early V3 marker gene *sim1a*, which activates the gene *slc17a6b* responsible for encoding the V3-specific glutamate neurotransmitter transporter [12,45]. However, there are differences between KA″ cells and V3 cells in terms of their differentiation process. While KA″ cells complete their differentiation by 24 hpf, V3 cells begin to emerge from LFP progenitor cells through either asymmetric division or no division at all, between 30 and 48 hpf. Despite this time shift, the same rules governing KA″ specification can be applied to V3 cells [7,9].

The differentiation of V3 cells from common LFP progenitor cells follows a sequential process, which is regulated by the amount, duration, and termination of Notch signaling. The level and duration of Notch activity determine the responsiveness to Shh, as discussed in the previous chapter, and ultimately influence the fate of these cells. LFP progenitors maintain high levels of Notch signaling to remain in their progenitor state, while V3 cells exhibit an intermediate level of Notch activity, and KA″ cells completely lack Notch response at 24 hpf. Sustained Notch signaling not only prevents the specification of KA″ and V3 interneurons but also expands the LFP domain. Contrariwise, blocking Notch signaling early, from 18 to 30 hpf, increases the number of V3 cells in the anterior part of the embryo, while the posterior regions mostly lack these interneurons. Meanwhile, KA″ cell numbers remain unchanged in the anterior regions, but a significant increase in KA″ cells is observed in the posterior parts of the spinal cord. These opposing changes in cell numbers can be explained by the fact that the spinal cord develops from anterior to posterior, with the anterior spinal cord being at a more advanced embryonic stage compared with the posterior spinal cord at a given time and that both KA″ and V3 interneurons develop from a common progenitor pool. Terminating constitutive Notch activity even earlier, at 14 hpf, results in an increase in KA″ cells at the expense of V3 interneurons. On the contrary, terminating constitutive Notch signaling at a later stage, specifically at 24 hpf, leads to the absence of KA″ cells and a significant increase in the number of V3 interneurons. These experiments highlight the significant impact of Notch signaling duration, rather than just the levels of Notch, on the specification of KA″ and V3 cells (Fig. 3) [9].

In their search for a Notch signaling mediator involved in this specification process, Jacobs *et al.* (2022) identified *Jag2b* through morpholino knockdown experiments. However, the absence of *Jag2b* must be compensated by *Jag2b*-independent processes that regulate the timing of KA″/V3 differentiation, as the specification of these cells did not occur prematurely despite the lack of *Jag2b*. This indicates that there may be other unknown external signals or pathways that could drive LFP specification, warranting further investigation [9].

ISN specification

Despite the discovery of the LFP as the origin of ISNs using scRNA-seq data analysis and knockdown experiments, the specific molecular mechanisms responsible for their development have remained unclear.

However, upon further analysis of the scRNA-seq data, it was found that the LFP progenitor cells exhibit strong expression of genes involved in the Notch signaling pathway, including the downstream targeted her genes. Interestingly, when the Notch pathway was inhibited between 2 and 3 dpf, there was an observed increase in the number of ISNs. These findings suggest that, alongside KA″ and V3 interneuron differentiation, the Notch signaling pathway may play a role in the specification of ISNs [13].

Neural specification—the case of the zebrafish p2 domain

V2 progenitor cells develop into three distinct V2 neuron subtypes

The p2 domain is one of 11 spinal cord precursor domains, located between the pMN and p1 domains (Fig. 1A) [17,78]. The p2 domains of zebrafish and mouse give rise to several distinct postmitotic subpopulations of V2 interneurons. In zebrafish, three subpopulations have been identified to date, termed V2a, V2b, and V2s (Figs 1B and 4).

V2a interneurons play an important role in locomotion by providing excitatory input to motor neurons [19,79]. They are characterized by the expression of *vsx1*, *vsx2*, and *lhx3* genes, and they are glutamatergic excitatory interneurons (Fig. 5) [14,19,80]. Studies have shown that V2a cells are circumferential descending interneurons (Fig. 4) that modulate swimming and escaping movements [14,81]. The TF gene *vsx1* has been identified as essential for the development of V2a interneurons, and its inhibition leads to an increase in V2b cells expressing *tal1*. Vsx1 represses *tal1* expression by directly binding to its cis-regulatory sequence (Table 2) [82].

V2b interneurons are inhibitory GABAergic neurons that are Ventral Lateral Descending interneurons (Fig. 4) [14,83]. The majority of V2b develops from a first intermediate progenitor pool of V2a/b, the rest are generated from a second intermediate progenitor pool of V2b/s (Fig. 5) [15]. V2b expresses *gata3*, *gata2a*, *tal1*, and *tal2* genes (Fig. 5) [16,83]. Studies have shown that V2b neurons have exclusive ipsilateral caudal innervations that connect directly to motor neurons, and they play a role in controlling tail beat frequency in zebrafish embryos [84,85]. *tal1* has been identified as the most important TF gene for the development of V2b cells and their GABAergic phenotype [83]. Loss-of-function studies have supported the crucial role of *tal1* in the development of V2b cells (Table 3) [83].

V2s are inhibitory glycinergic interneurons that develop from an intermediate progenitor pool of V2b and V2s [16]. They express *sox1a*, *sox1b*, and *nkx1.2lb* and have long ipsilateral descending axonal projections with a short branch at the ventral midline (Fig. 5,

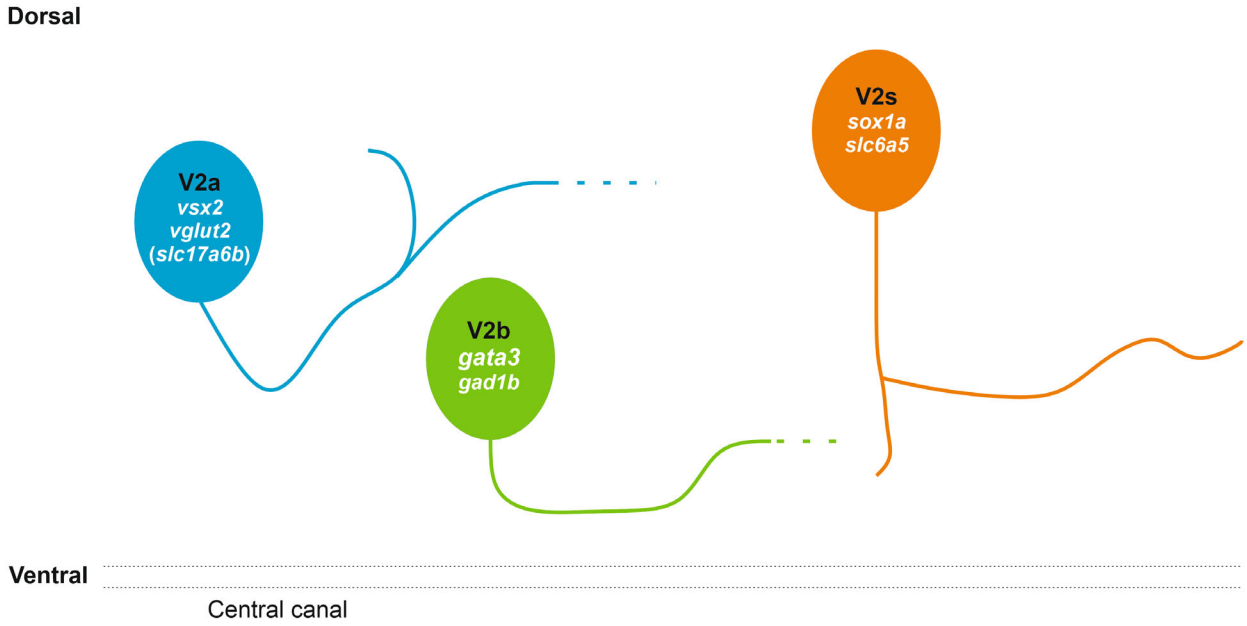

Fig. 4. p2 domain gives rise to three functionally distinct V2 neuron subtypes known as V2a, V2b, and V2s. V2a interneurons (blue) are glutamatergic interneurons with ipsilateral excitatory synapses. V2b neurons (green) are GABAergic and their projections are mainly ipsilateral, with V2b axons descending toward the caudal end of the spinal cord. V2s neurons (orange) are glycinergic and have long ipsilateral descending axonal projections with a short branch at the ventral midline.

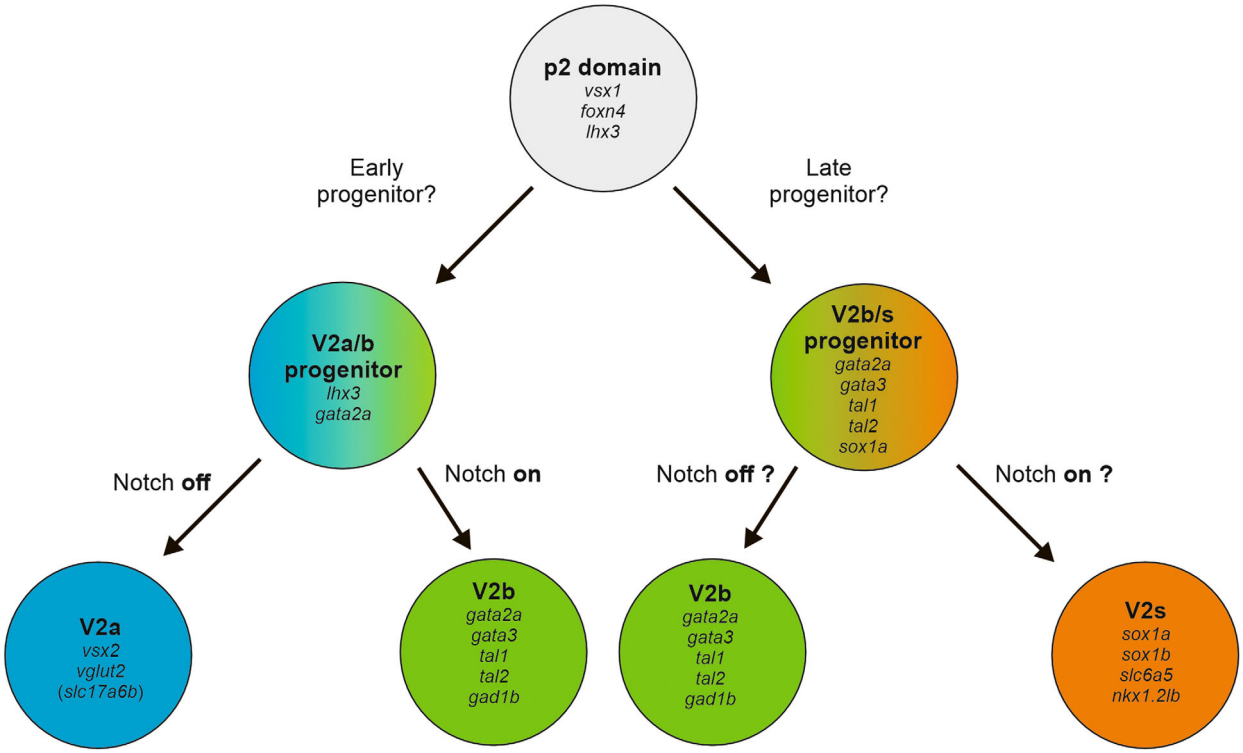

Fig. 5. Model of V2 subtype development from two distinct p2 intermediate progenitor pools. (Left) The first pool produces sister cells with different levels of Notch activity, leading to differentiation into V2b and V2a neurons. (Right) The second pool generates V2b and V2s neurons, with the decision between V2b and V2s also depending on Notch signaling. The first group of p2 intermediate progenitors is probably generated earlier than the second group of p2 intermediate progenitors. The decision between V2b and V2s is also dependent on Notch signaling, but the mechanism of action of this signaling is not yet well-documented.

Table 2. Characteristics of zebrafish V2 cells.

	P2 progenitor	V2a/b progenitor	V2b/s progenitor	V2a	V2b	V2s
Marker genes	*vsx1* *foxn4* *lhx3*	*lhx3* *gata2a*	*gata2a* *gata3* *tal1* *tal2* *sox1a*	*vsx2* (*chx10*) *vsx1* *vglut2* (*slc17a6b*)	*gata2a* *gata3* *tal1* *tal2* *gad1b* (*GAD67*)	*sox1a* *sox1b* *nkx1.2 lb* *slc6a5*
Neurotransmitter	–	–	–	Glutamatergic	GABAergic	Glycinergic
Excitatory/ inhibitory	–	–	–	Excitatory	Inhibitory	Inhibitory
Anatomy	–	–	–	Circumferential descending	Ventral lateral descending	Ipsilaterally descending

Table 2) [16]. V2s are believed to develop later than V2a and most V2b and become fully glycinergic during the second day of embryonic development. Studies have shown that *sox1a* and *sox1b* expression and function are critical determinants for the formation and specification of V2s interneurons (Table 3) [16]. In *sox1a* and *sox1b* double mutants, there is a clear reduction of V2s at the expense of V2b, indicating the importance of these genes in the development of V2s (Table 3). Zebrafish V2s [16] and mouse V2c [21] are likely to be homologous (Table 4). The differentiation of both cell types depends on Sox1 activity, and they develop late from a common precursor cell type, V2b/c in mouse and V2b/s in zebrafish (Fig. 5) [15,16,21].

Zebrafish V2a, V2b, and V2s neurons are close to each other, intermingled, and have all ipsilateral

Table 3. Zebrafish mutants with a V2 neuron phenotype.

Target	Knock down/knock out	How are V2 cells affected?	References
vsx1	• Knock down: Splice blocking morpholino antisense oligonucleotide • Knock out: CRISPR-Cas9	• Decreased number of excitatory V2a cells (*slc17a6b;vsx2*-positive) • Increased number of *tal1*-expressing V2 cells • Increased number of inhibitory V2b cells (*gad1b;tal1*-positive) • No effect on V2s cells marker (*sox1a* and *sox1b*) • In *vsx1* overexpression, increased number of *vsx2*, decreased number of *tal1*-expressing cells	[82]
tal1	• Knock out: ENU-induced mutagenesis	• Lost expression of *pkd2l1* in V2 cells • Reduced number of GABAergic cells (*gad1b* expression) • Reduced number of *sox1a* cells • No *tal2* expression • Almost no cells expressing *sox1b* • Normal number of *gata2a* and *gata3*-expressing cells	[83]
gata2a	• Knock out: Zinc-finger nuclease	• Reduced expression of *pkd2l1* in V2 cells • Reduced number of GABAergic cells (*gad1b* expression) • No difference in the number of *tal1* or *tal2*-expressing V2 cells • Slightly decreased number of *gata3* cells	[83]
gata3	• Knock out: Zinc-finger nuclease	• Reduced number of GABAergic cells (*gad1b* expression) • Decreased number of *gata2a* and *tal1*	[83]
gata2a; *gata3*	• Knock out: Zinc-finger nuclease	• Strong reduction in the number of GABAergic cells (*gad1b* expression) compare with single mutants	[83]
sox1a; *sox1b*	• Knock down: Splice blocking morpholino antisense oligonucleotide • Knock out: CRISPR-Cas9	• Increased number of V2b cells marker (*gata2a, gata3, tal1* and *tal2*) in spinal cord • Reduced number of glycinergic marker (*slc6a5*) • No difference in the number of V2a cell marker (*vsx1* and *vsx2*)	[16]
Mind bomb (ubiquitin ligase)	• Knock out	• Reduction in the number of V2b at the expense of V2a • Loss of GABAergic cells in V2 domain	[14]

descending projections, but they receive information from different sources, connect to different targets and are not connected to each other (Fig. 4) [15,84].

How are functionally distinct V2 neurons generated from molecularly identical progenitor cells?

V2a, V2b, and V2s neurons are generated from progenitor cells in the p2 domain of the spinal cord (Fig. 5) [14,16,19]. p2 cells are located in the ventricular zone, are highly proliferative, and undergo multiple divisions. Some of these cells exit the cell cycle, become postmitotic, and move laterally. They begin to express a specific combination of TF genes, which ultimately results in the development of mature, functionally distinct neurons [17,24].

The early p2 domain is characterized by the expression of *irx3a*, *nkx6.1*, and *nkx6.2* genes (Fig. 5, Table 2) [86,87]. Progenitor cells within the p2 domain express several TF genes, such as *vsx1*, *lhx3*, and *foxn4* (Fig. 5, Table 2) [14,19,88]. After the final division, some p2 cells generate two sister cells that differentiate into distinct functional cell types. The sister cell groups responsible for giving rise to V2a and V2b are referred to as V2a/b intermediate progenitor cells (Fig. 5) [19]. Another population of intermediate progenitors, known as V2b/s, has also been identified and develops into V2b and V2s neurons (Fig. 5) [15,16]. V2a/b intermediate progenitor cells produce approximately 75% of mature V2 neurons, while V2b/s cells contribute to only 25% [15]. It is suggested that V2b/s intermediate progenitor cells may either develop after V2a/b cells, or their specification into V2b and V2s neurons occurs at a later stage, as zebrafish V2s neurons tend to develop later compared to the majority of V2b and V2a neurons (Fig. 5) [15,16].

Molecular mechanisms of V2a, V2b, and V2s specification

Research conducted on mouse and zebrafish models has demonstrated that the Notch signaling pathway plays a vital and conserved role in determining the

Table 4. Comparative table between mouse and zebrafish interneurons.

Organisms	Type of interneuron	Developmental stage birth date and origin	Pathway involved in generation/ specification	Marker genes	Function	References
Zebrafish	KA′ (dorsal)	10–15 hpf from pMN	Shh	*sst1.1* *gads* *pkd2l1*	Detects ipsilateral spinal bending, transmits mechanosensory input to locomotor network for slow swimming and postural control	[12,48,49,100,101]
Mouse	CSF-cN′ (dorsal)	E13–E14 from late p2 domain and dorsal half of pOL domain	Shh	*Pkd2l1* *GAD*	Enhance locomotor speed	[94,102,103]
Zebrafish	KA″ (ventral)	10–24 hpf from progenitors of LFP	Shh and Notch	*urp1* *gads* *pkd2l1*	Detects longitudinal spinal bending, transmits mechanosensory input to locomotor network for fast swimming and postural control	[9,11,12,55,100,101,104]
Mouse	CSF-cN″ (ventral)	E13–E14 from p3 progenitors adjacent to the floor plate	Shh	*Pkd2l1* *GAD*	Enhance locomotor speed	[94,102,103]
Zebrafish	V3	24–48 hpf from late LFP that express *shh*	Shh and Notch	*sim1a* *slc17a6b (vglut2a)*	*In vivo* fictive swimming, locomotor amplitude	[12,17,60,105–107]
Mouse	V3	E9.0–E12 from p3 domain	Shh	*Sim1* *Vglut2*	Establishment of robust motor rhythm during walking	[59,108–112]
Zebrafish	ISN	2.5–3 dpf from late progenitor of LFP	Shh and Notch	*fev1 (pet1)* *tph2*	Beat-and-glide swimming, spinal cord injury regeneration and motor circuitry reinstitution	[10,13,51,74,113]
Mouse	ISN	E16.5 from progenitors adjacent to the floor plate, located dorsal to the central canal	Shh	*Pet1* *Tph2*	Modulation of autonomic functions, fictive locomotor activity	[114–119]
Zebrafish	V2a	17 hpf from p2a/b progenitors	Notch off	*vsx2* *vglut2*	Involved in control movement amplitude and ensuring left–right alternation at higher frequencies of swimming	[14,19,80,81]
Mouse	V2a	P2a/b progenitors	Notch off	*Vsx2 (CHX10)* *Vglut2*	Involved in reach-and-grasp movement	[20,89,97,120–122]
Zebrafish	V2b	17 hpf from p2a/b progenitors	Notch on	*gata3* *gads*	Involved in the ipsilateral inhibition in speed control	[14,19,84,91]
Mouse	V2b	E10–E12 from p2a/b progenitors	Dll4-mediated Notch, BMP/ TGFβ	*Gata3* *GAD1*	Involved in flexor/extensor alternation	[20,89,92,120,123–125]
Zebrafish	V2s	24 hpf from p2b/s progenitors	Notch on	*sox1a* *sox1b* *slc6a5*	Involved in fast escape movement	[15,16,84]
Mouse	V2c	P2b/c progenitors	Notch on	*Sox1* *Slc6a5*	Unknown	[21]

development of V2a and V2b cells from the intermediate progenitor pool of V2a/b cells [14,19,20,89–92].

In zebrafish mutants that lack *Mind bomb1* (*Mib1*), the absence of Notch signaling leads to an increase in V2a cells at the expense of V2b cells. These findings indicate that during the normal specification process, it is crucial to deactivate Notch signaling in V2a progenitors while maintaining its activation in V2b progenitors (Table 3) [14]. The precise contribution of different zebrafish membrane-bound Notch ligands (DeltaA, DeltaC, and DeltaD) and Notch receptors (Notch1a, Notch1b, and Notch3) in this process has been investigated, revealing that different combinations of Notch ligands and receptors play distinct roles in the development of V2 interneurons in zebrafish. Specifically, DeltaA and DeltaD, activated by the *ubiquitin ligase Mib1*, work together to maintain p2 progenitors, while DeltaA and DeltaC redundantly determine the fate of V2a/V2b cells. Notch1a, Notch1b, and Notch3 are involved in p2 progenitor proliferation, while Notch1a primarily contributes to the specification of V2a/V2b cells [90]. Recently, a downstream effector of the Notch pathway called *her15.1* has been discovered to exhibit differential expression in the V2 lineage. The early and transient expression of *her15.1* in immature V2b cells is crucial for the specification of V2b neurons. Conversely, in mature neurons, the downregulation of *her15.1* is necessary for the initiation of axon genesis in the V2b neurons [91]. However, it remains unclear which specific Notch genes regulate the activity of *her15.1* and the precise roles of other zebrafish *her* genes in this process. Additionally, the combination of Notch and Delta molecules that function upstream of *her15.1* activity has yet to be determined.

Another V2 intermediate progenitor group differentiates into V2b and V2s neurons [15,16], and Notch signaling is crucial for their development. Pharmacological inhibition of the Notch pathway with LY411575 chemical between 16 and 24 hpf results in an increase in the number of V2b and a reduction in the number of V2s, suggesting that Notch needs to be turned on in V2s precursors and off in V2b for the correct specification of V2b and V2s [16]. However, it is not clear why Notch signaling is required for V2b specification in V2a/b progenitor population but not in V2b/s. One possibility is that there are intrinsic molecular differences between these two V2b populations that determine their sensitivity to Notch signaling. Alternatively, the difference may be related to the temporal regulation of Notch signaling during the development of these two populations. Nevertheless, the specific Notch receptors, ligands, and effectors involved in V2s specification remain unknown. While Notch signaling has been implicated in V2 specification in zebrafish, other developmental pathways such as BMP, fibroblast growth factor, and Shh signaling pathways have also been associated with neuronal development. Further research is necessary to identify the specific signaling pathways, including Notch components, involved in V2a, V2b, and V2s cell specification.

Commonalities and differences in the specification of neurons between the LFP and p2 progenitor domains

At first glance, the processes of development and specification of p2 domain and LFP domain neurons appear to differ. As mentioned earlier in this review, LFP progenitor cells sequentially generate three distinct populations of interneurons at different stages of embryonic development. In contrast, in zebrafish, the p2 domain was shown to generate only several subpopulations of V2 interneurons. However, upon closer examination of the processes and pathways involved in the specification of the LFP and p2 domains, it becomes clear that very similar mechanisms are used to generate distinct populations from the two progenitor domains. First, the generation of distinct V2a, V2b, and V2s interneurons also occurs sequentially and at different embryonic stages, with mature V2a interneurons and most V2b interneurons being found in the spinal cord earlier than V2s [15,16]. Second, the specification of different V2 subpopulations, as well as distinct LFP neurons, relies on the Notch signaling pathway. Third, in mouse, the p3 population gives rise to several V3 subtypes, namely V3.1, V3.2, V3.3, and V3.4 [61,93]. These V3 subtypes probably result from further specification of the p3 domain into different subtypes, similar to what is observed in the p2 progenitor domain [14–16,18,20–23]. Furthermore, in mouse, some CSF-cNs (homologous to zebrafish KA′) were shown to be generated late from the p2 progenitor domain [94], indicating that, at least in mouse, the p2 progenitor domain gives rise not only to different V2 subtypes but also to distinct neuronal populations at different periods of embryonic development. It is still not known whether this is a mouse-specific feature of the p2 domain or if it is common to other vertebrates such as the zebrafish.

In summary, although the development and specification of p2 domain and LFP domain neurons may initially appear distinct, closer examination reveals that they share similar mechanisms. Sequential generation of specific interneuron populations, involvement of the Notch signaling pathway, and subsequent specification into subtypes are common features observed in both domains.

Unveiling the potential of zebrafish model in spinal interneuron research

Numerous studies have presented compelling evidence indicating a remarkable similarity in the key classes of spinal interneurons involved in movement control, gene expression patterns, and transcriptional gene regulatory networks responsible for establishing the spinal neuronal circuitry between zebrafish and mouse (Table 4) [17,24,95,96]. Despite the significant evolutionary distance between vertebrates, this parallelism suggests a shared basis in their spinal neuronal circuitry. This indicates a highly conserved mechanism of spinal cord development among vertebrates, specifically applying to CSF-cNs (KA), V3, ISNs, and all three subsets of V2 interneurons discussed in this review. Table 4 provides a summary of the developmental and functional aspects of these cells in both mouse and zebrafish models. The Zebrafish offers several advantages as a model organism, including advanced genetic tools, high regenerative capacity, optical transparency of embryos, and short generation time. These attributes provide significant potential for exploring the complexities of spinal circuitry analysis. Researchers can now leverage the strengths of the zebrafish model in conjunction with cutting-edge genomic techniques such as CAGE-seq, ATAC-seq, ChIP-seq, bulk, and scRNA-seq. This combined approach enables the unveiling of crucial insights into the development and function of spinal interneurons. This comprehensive understanding of spinal cord development and diversity bears profound implications for the advancement of targeted therapies for neurological disorders and spinal cord injuries. Encouraging studies have already demonstrated the successful generation of functional V2a cells in cell culture [97] and the development of dorsoventral human spinal cord organoids [98], highlighting the potential of this research in clinical applications.

Acknowledgements

We thank F. Chen, T. Beil, A. Schröck, L. Yang, and V. Gerber for producing data that inspired this review article. The research in Sepand Rastegar's laboratory is supported by the Helmholtz Association BioInterfaces in Technology and Medicine and Natural, Artificial, and Cognitive Information Processing (NACIP) Programs and by project grants of the German Research Foundation (the Deutsche Forschungsgemeinschaft) GRK2039, STR 439/17-1, and RA 3469/5-1. Open Access funding enabled and organized by Projekt DEAL. Open Access funding enabled and organized by Projekt DEAL.

Conflict of interest

The authors declare no conflict of interest.

Author contributions

GC, MK, SP, and SR wrote the review. SR coordinated the process. All authors contributed to the article and approved the submitted version.

References

1 Dessaud E, McMahon AP & Briscoe J (2008) Pattern formation in the vertebrate neural tube: a sonic hedgehog morphogen-regulated transcriptional network. *Development* **135**, 2489–2503.
2 Sagner A & Briscoe J (2019) Establishing neuronal diversity in the spinal cord: a time and a place. *Development* **146**, dev182154.
3 Sagner A & Briscoe J (2017) Morphogen interpretation: concentration, time, competence, and signaling dynamics. *Wiley Interdiscip Rev Dev Biol* **6**, e271.
4 Le Dreau G & Marti E (2012) Dorsal-ventral patterning of the neural tube: a tale of three signals. *Dev Neurobiol* **72**, 1471–1481.
5 Andrews MG, Kong J, Novitch BG & Butler SJ (2019) New perspectives on the mechanisms establishing the dorsal-ventral axis of the spinal cord. *Curr Top Dev Biol* **132**, 417–450.
6 Danesin C & Soula C (2017) Moving the Shh source over time: what impact on neural cell diversification in the developing spinal cord? *J Dev Biol* **5**, 4.
7 Huang P, Xiong F, Megason SG & Schier AF (2012) Attenuation of Notch and Hedgehog signaling is required for fate specification in the spinal cord. *PLoS Genet* **8**, e1002762.
8 Jacobs CT & Huang P (2019) Notch signalling maintains Hedgehog responsiveness via a Gli-dependent mechanism during spinal cord patterning in zebrafish. *Elife* **8**, e49252.
9 Jacobs CT, Kejriwal A, Kocha KM, Jin KY & Huang P (2022) Temporal cell fate determination in the spinal cord is mediated by the duration of Notch signalling. *Dev Biol* **489**, 1–13.
10 Montgomery JE, Wahlstrom-Helgren S, Wiggin TD, Corwin BM, Lillesaar C & Masino MA (2018) Intraspinal serotonergic signaling suppresses locomotor activity in larval zebrafish. *Dev Neurobiol* **78**, 807–827.
11 Schäfer M, Kinzel D & Winkler C (2007) Discontinuous organization and specification of the lateral floor plate in zebrafish. *Dev Biol* **301**, 117–129.
12 Yang L, Rastegar S & Strahle U (2010) Regulatory interactions specifying Kolmer-Agduhr interneurons. *Development* **137**, 2713–2722.

13 Chen F, Köhler M, Cucun G, Takamiya M, Kizil C, Cosacak MI & Rastegar S (2023) sox1a:eGFP transgenic line and single-cell transcriptomics reveal the origin of zebrafish intraspinal serotonergic neurons. *iScience* **26**, 107342.
14 Batista MF, Jacobstein J & Lewis KE (2008) Zebrafish V2 cells develop into excitatory CiD and Notch signalling dependent inhibitory VeLD interneurons. *Dev Biol* **322**, 263–275.
15 Bello-Rojas S & Bagnall MW (2022) Clonally related, Notch-differentiated spinal neurons integrate into distinct circuits. *Elife* **11**, e83680.
16 Gerber V, Yang L, Takamiya M, Ribes V, Gourain V, Peravali R, Stegmaier J, Mikut R, Reischl M, Ferg M *et al.* (2019) The HMG box transcription factors Sox1a and Sox1b specify a new class of glycinergic interneuron in the spinal cord of zebrafish embryos. *Development* **146**, dev172510.
17 Goulding M (2009) Circuits controlling vertebrate locomotion: moving in a new direction. *Nat Rev Neurosci* **10**, 507–518.
18 Karunaratne A, Hargrave M, Poh A & Yamada T (2002) GATA proteins identify a novel ventral interneuron subclass in the developing chick spinal cord. *Dev Biol* **249**, 30–43.
19 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.
20 Li S, Misra K, Matise MP & Xiang M (2005) Foxn4 acts synergistically with Mash1 to specify subtype identity of V2 interneurons in the spinal cord. *Proc Natl Acad Sci USA* **102**, 10688–10693.
21 Panayi H, Panayiotou E, Orford M, Genethliou N, Mean R, Lapathitis G, Li S, Xiang M, Kessaris N, Richardson WD *et al.* (2010) Sox1 is required for the specification of a novel p2-derived interneuron subtype in the mouse ventral spinal cord. *J Neurosci* **30**, 12274–12280.
22 Smith E, Hargrave M, Yamada T, Begley CG & Little MH (2002) Coexpression of SCL and GATA3 in the V2 interneurons of the developing mouse spinal cord. *Dev Dyn* **224**, 231–237.
23 Zhou Y, Yamamoto M & Engel JD (2000) GATA2 is required for the generation of V2 interneurons. *Development* **127**, 3829–3838.
24 Lewis KE (2006) How do genes regulate simple behaviours? Understanding how different neurons in the vertebrate spinal cord are genetically specified. *Philos Trans R Soc Lond B Biol Sci* **361**, 45–66.
25 Jacobs CT & Huang P (2021) Complex crosstalk of Notch and Hedgehog signalling during the development of the central nervous system. *Cell Mol Life Sci* **78**, 635–644.
26 Odenthal J & Nusslein-Volhard C (1998) Fork head domain genes in zebrafish. *Dev Genes Evol* **208**, 245–258.
27 Odenthal J, van Eeden FJ, Haffter P, Ingham PW & Nusslein-Volhard C (2000) Two distinct cell populations in the floor plate of the zebrafish are induced by different pathways. *Dev Biol* **219**, 350–363.
28 Strahle U, Lam CS, Ertzer R & Rastegar S (2004) Vertebrate floor-plate specification: variations on common themes. *Trends Genet* **20**, 155–162.
29 Appel B, Fritz A, Westerfield M, Grunwald DJ, Eisen JS & Riley BB (1999) Delta-mediated specification of midline cell fates in zebrafish embryos. *Curr Biol* **9**, 247–256.
30 Le Douarin NM & Halpern ME (2000) Discussion point. Origin and specification of the neural tube floor plate: insights from the chick and zebrafish. *Curr Opin Neurobiol* **10**, 23–30.
31 Schafer M, Rembold M, Wittbrodt J, Schartl M & Winkler C (2005) Medial floor plate formation in zebrafish consists of two phases and requires trunk-derived Midkine-a. *Genes Dev* **19**, 897–902.
32 Row RH, Tsotras SR, Goto H & Martin BL (2016) The zebrafish tailbud contains two independent populations of midline progenitor cells that maintain long-term germ layer plasticity and differentiate in response to local signaling cues. *Development* **143**, 244–254.
33 Kennedy TE, Serafini T, de la Torre JR & Tessier-Lavigne M (1994) Netrins are diffusible chemotropic factors for commissural axons in the embryonic spinal cord. *Cell* **78**, 425–435.
34 Lauderdale JD, Davis NM & Kuwada JY (1997) Axon tracts correlate with netrin-1a expression in the zebrafish embryo. *Mol Cell Neurosci* **9**, 293–313.
35 Charron F, Stein E, Jeong J, McMahon AP & Tessier-Lavigne M (2003) The morphogen sonic hedgehog is an axonal chemoattractant that collaborates with netrin-1 in midline axon guidance. *Cell* **113**, 11–23.
36 Schauerte HE, van Eeden FJ, Fricke C, Odenthal J, Strahle U & Haffter P (1998) Sonic hedgehog is not required for the induction of medial floor plate cells in the zebrafish. *Development* **125**, 2983–2993.
37 Chen W, Burgess S & Hopkins N (2001) Analysis of the zebrafish smoothened mutant reveals conserved and divergent functions of hedgehog activity. *Development* **128**, 2385–2396.
38 Varga ZM, Amores A, Lewis KE, Yan YL, Postlethwait JH, Eisen JS & Westerfield M (2001) Zebrafish smoothened functions in ventral neural tube specification and axon tract formation. *Development* **128**, 3497–3509.
39 Karlstrom RO, Tyurina OV, Kawakami A, Nishioka N, Talbot WS, Sasaki H & Schier AF (2003) Genetic analysis of zebrafish gli1 and gli2 reveals divergent

requirements for gli genes in vertebrate development. *Development* **130**, 1549–1564.
40 Schafer M, Kinzel D, Neuner C, Schartl M, Volff JN & Winkler C (2005) Hedgehog and retinoid signalling confines nkx2.2b expression to the lateral floor plate of the zebrafish trunk. *Mech Dev* **122**, 43–56.
41 Fontenas L & Kucenas S (2018) Motor exit point (MEP) glia: novel myelinating glia that bridge CNS and PNS myelin. *Front Cell Neurosci* **12**, 333.
42 Fontenas L & Kucenas S (2021) Spinal cord precursors utilize neural crest cell mechanisms to generate hybrid peripheral myelinating glia. *Elife* **10**, e64267.
43 Kucenas S, Takada N, Park HC, Woodruff E, Broadie K & Appel B (2008) CNS-derived glia ensheath peripheral nerves and mediate motor root development. *Nat Neurosci* **11**, 143–151.
44 Djenoune L & Wyart C (2017) Light on a sensory interface linking the cerebrospinal fluid to motor circuits in vertebrates. *J Neurogenet* **31**, 113–127.
45 Yang L, Wang F & Strahle U (2020) The genetic programs specifying Kolmer-Agduhr interneurons. *Front Neurosci* **14**, 577879.
46 Bernhardt RR, Patel CK, Wilson SW & Kuwada JY (1992) Axonal trajectories and distribution of GABAergic spinal neurons in wildtype and mutant zebrafish lacking floor plate cells. *J Comp Neurol* **326**, 263–272.
47 Roberts A & Clarke JD (1982) The neuroanatomy of an amphibian embryo spinal cord. *Philos Trans R Soc Lond B Biol Sci* **296**, 195–212.
48 Shin J, Poling J, Park HC & Appel B (2007) Notch signaling regulates neural precursor allocation and binary neuronal fate decisions in zebrafish. *Development* **134**, 1911–1920.
49 Park HC, Shin J & Appel B (2004) Spatial and temporal regulation of ventral spinal cord precursor specification by Hedgehog signaling. *Development* **131**, 5959–5969.
50 Zheng PF, Xie HB, Zhu PP & Zhao CT (2022) Distribution pattern of floor plate neurons in zebrafish. *Yi Chuan* **44**, 510–520.
51 Montgomery JE, Wiggin TD, Rivera-Perez LM, Lillesaar C & Masino MA (2016) Intraspinal serotonergic neurons consist of two, temporally distinct populations in developing zebrafish. *Dev Neurobiol* **76**, 673–687.
52 Quan FB, Dubessy C, Galant S, Kenigfest NB, Djenoune L, Leprince J, Wyart C, Lihrmann I & Tostivint H (2015) Comparative distribution and in vitro activities of the urotensin II-related peptides URP1 and URP2 in zebrafish: evidence for their colocalization in spinal cerebrospinal fluid-contacting neurons. *PLoS One* **10**, e0119290.
53 Delmas P (2004) Polycystins: from mechanosensation to gene regulation. *Cell* **118**, 145–148.
54 Orts-Del'immagine A, Wanaverbecq N, Tardivel C, Tillement V, Dallaporta M & Trouslard J (2012) Properties of subependymal cerebrospinal fluid contacting neurones in the dorsal vagal complex of the mouse brainstem. *J Physiol* **590**, 3719–3741.
55 Bohm UL, Prendergast A, Djenoune L, Nunes Figueiredo S, Gomez J, Stokes C, Kaiser S, Suster M, Kawakami K, Charpentier M *et al.* (2016) CSF-contacting neurons regulate locomotion by relaying mechanical stimuli to spinal circuits. *Nat Commun* **7**, 10866.
56 Sternberg JR, Prendergast AE, Brosse L, Cantaut-Belarif Y, Thouvenin O, Orts-Del'Immagine A, Castillo L, Djenoune L, Kurisu S, McDearmid JR *et al.* (2018) Pkd2l1 is required for mechanoception in cerebrospinal fluid-contacting neurons and maintenance of spine curvature. *Nat Commun* **9**, 3804.
57 Hubbard JM, Bohm UL, Prendergast A, Tseng PB, Newman M, Stokes C & Wyart C (2016) Intraspinal sensory neurons provide powerful inhibition to motor circuits ensuring postural control during locomotion. *Curr Biol* **26**, 2841–2853.
58 Orts-Del'Immagine A, Cantaut-Belarif Y, Thouvenin O, Roussel J, Baskaran A, Langui D, Koeth F, Bivas P, Lejeune FX, Bardet PL *et al.* (2020) Sensory neurons contacting the cerebrospinal fluid require the Reissner fiber to detect spinal curvature in vivo. *Curr Biol* **30**, 827–839.e4.
59 Zhang Y, Narayan S, Geiman E, Lanuza GM, Velasquez T, Shanks B, Akay T, Dyck J, Pearson K, Gosgnach S *et al.* (2008) V3 spinal neurons establish a robust and balanced locomotor rhythm during walking. *Neuron* **60**, 84–96.
60 Wiggin TD, Montgomery JE, Brunick AJ, Peck JH & Masino MA (2022) V3 interneurons are active and recruit spinal motor neurons during in vivo fictive swimming in larval zebrafish. *eNeuro* **9**, ENEURO.0476-21.2022.
61 Deska-Gauthier D, Borowska-Fielding J, Jones CT & Zhang Y (2020) The temporal neurogenesis patterning of spinal p3-V3 interneurons into divergent subpopulation assemblies. *J Neurosci* **40**, 1440–1452.
62 Carlsson A, Magnusson T & Rosengren E (1963) 5-Hydroxytryptamine of the spinal cord normally and after transection. *Experientia* **19**, 359.
63 Anden NE, Haeggendal J, Magnusson T & Rosengren E (1964) The time course of the disappearance of noradrenaline and 5-hydroxytryptamine in the spinal cord after transection. *Acta Physiol Scand* **62**, 115–118.
64 Shibuya T & Anderson EG (1968) The influence of chronic cord transection on the effects of 5-hydroxytryptophan, l-tryptophan and pargyline on

spinal neuronal activity. *J Pharmacol Exp Ther* **164**, 185–190.
65 Clineschmidt BV, Pierce JE & Lovenberg W (1971) Tryptophan hydroxylase and serotonin in spinal cord and brain stem before and after chronic transection. *J Neurochem* **18**, 1593–1596.
66 McLean DL & Fetcho JR (2004) Ontogeny and innervation patterns of dopaminergic, noradrenergic, and serotonergic neurons in larval zebrafish. *J Comp Neurol* **480**, 38–56.
67 Brustein E, Chong M, Holmqvist B & Drapeau P (2003) Serotonin patterns locomotor network activity in the developing zebrafish by modulating quiescent periods. *J Neurobiol* **57**, 303–322.
68 Hendricks T, Francis N, Fyodorov D & Deneris ES (1999) The ETS domain factor Pet-1 is an early and precise marker of central serotonin neurons and interacts with a conserved element in serotonergic genes. *J Neurosci* **19**, 10348–10356.
69 Walther DJ, Peter JU, Bashammakh S, Hortnagl H, Voits M, Fink H & Bader M (2003) Synthesis of serotonin by a second tryptophan hydroxylase isoform. *Science* **299**, 76.
70 Schmidt BJ & Jordan LM (2000) The role of serotonin in reflex modulation and locomotor rhythm production in the mammalian spinal cord. *Brain Res Bull* **53**, 689–710.
71 Lillesaar C (2011) The serotonergic system in fish. *J Chem Neuroanat* **41**, 294–308.
72 Van Raamsdonk W, Bosch TJ, Smit-Onel MJ & Maslam S (1996) Organisation of the zebrafish spinal cord: distribution of motoneuron dendrites and 5-HT containing cells. *Eur J Morphol* **34**, 65–77.
73 Van Dongen PA, Hokfelt T, Grillner S, Verhofstad AA & Steinbusch HW (1985) Possible target neurons of 5-hydroxytryptamine fibers in the lamprey spinal cord: immunohistochemistry combined with intracellular staining with Lucifer yellow. *J Comp Neurol* **234**, 523–535.
74 Huang CX, Zhao Y, Mao J, Wang Z, Xu L, Cheng J, Guan NN & Song J (2021) An injury-induced serotonergic neuron subpopulation contributes to axon regrowth and function restoration after spinal cord injury in zebrafish. *Nat Commun* **12**, 7093.
75 Xu J, Srinivas BP, Tay SY, Mak A, Yu X, Lee SG, Yang H, Govindarajan KR, Leong B, Bourque G *et al.* (2006) Genomewide expression profiling in the zebrafish embryo identifies target genes regulated by Hedgehog signaling during vertebrate development. *Genetics* **174**, 735–752.
76 Matise MP, Epstein DJ, Park HL, Platt KA & Joyner AL (1998) Gli2 is required for induction of floor plate and adjacent cells, but not most ventral neurons in the mouse central nervous system. *Development* **125**, 2759–2770.
77 Jacob J & Briscoe J (2003) Gli proteins and the control of spinal-cord patterning. *EMBO Rep* **4**, 761–765.
78 Briscoe J & Ericson J (2001) Specification of neuronal fates in the ventral neural tube. *Curr Opin Neurobiol* **11**, 43–49.
79 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.
80 Kimura Y, Okamura Y & Higashijima S (2006) alx, a zebrafish homolog of Chx10, marks ipsilateral descending excitatory interneurons that participate in the regulation of spinal locomotor circuits. *J Neurosci* **26**, 5684–5697.
81 Menelaou E & McLean DL (2019) Hierarchical control of locomotion by distinct types of spinal V2a interneurons in zebrafish. *Nat Commun* **10**, 4197.
82 Zhang Q, Xu H, Zhao W, Zheng J, Sun L & Luo C (2020) Zygotic Vsx1 plays a key role in defining V2a interneuron sub-lineage by directly repressing tal1 transcription in zebrafish. *Int J Mol Sci* **21**, 3600.
83 Andrzejczuk LA, Banerjee S, England SJ, Voufo C, Kamara K & Lewis KE (2018) Tal1, Gata2a, and Gata3 have distinct functions in the development of V2b and cerebrospinal fluid-contacting KA spinal neurons. *Front Neurosci* **12**, 170.
84 Callahan RA, Roberts R, Sengupta M, Kimura Y, Higashijima SI & Bagnall MW (2019) Spinal V2b neurons reveal a role for ipsilateral inhibition in speed control. *Elife* **8**, e47837.
85 Sengupta M, Daliparthi V, Roussel Y, Bui TV & Bagnall MW (2021) Spinal V1 neurons inhibit motor targets locally and sensory targets distally. *Curr Biol* **31**, 3820–3833.e4.
86 England S, Batista MF, Mich JK, Chen JK & Lewis KE (2011) Roles of Hedgehog pathway components and retinoic acid signalling in specifying zebrafish ventral spinal cord neurons. *Development* **138**, 5121–5134.
87 Lupo G, Harris WA & Lewis KE (2006) Mechanisms of ventral patterning in the vertebrate nervous system. *Nat Rev Neurosci* **7**, 103–114.
88 Seredick S, Hutchinson SA, Van Ryswyk L, Talbot JC & Eisen JS (2014) Lhx3 and Lhx4 suppress Kolmer-Agduhr interneuron characteristics within zebrafish axial motoneurons. *Development* **141**, 3900–3909.
89 Del Barrio MG, Taveira-Marques R, Muroyama Y, Yuk DI, Li S, Wines-Samuelson M, Shen J, Smith HK, Xiang M, Rowitch D *et al.* (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.

90 Okigawa S, Mizoguchi T, Okano M, Tanaka H, Isoda M, Jiang YJ, Suster M, Higashijima S, Kawakami K & Itoh M (2014) Different combinations of Notch ligands and receptors regulate V2 interneuron progenitor proliferation and V2a/V2b cell fate determination. *Dev Biol* **391**, 196–206.
91 Mizoguchi T, Fukada M, Iihama M, Song X, Fukagawa S, Kuwabara S, Omaru S, Higashijima SI & Itoh M (2020) Transient activation of the Notch-her15.1 axis plays an important role in the maturation of V2b interneurons. *Development* **147**, dev191312.
92 Misra K, Luo H, Li S, Matise M & Xiang M (2014) Asymmetric activation of Dll4-Notch signaling by Foxn4 and proneural factors activates BMP/TGFbeta signaling to specify V2b interneurons in the spinal cord. *Development* **141**, 187–198.
93 Delile J, Rayon T, Melchionda M, Edwards A, Briscoe J & Sagner A (2019) Single cell transcriptomics reveals spatial and temporal dynamics of gene expression in the developing mouse spinal cord. *Development* **146**, dev173807.
94 Petracca YL, Sartoretti MM, Di Bella DJ, Marin-Burgin A, Carcagno AL, Schinder AF & Lanuza GM (2016) The late and dual origin of cerebrospinal fluid-contacting neurons in the mouse spinal cord. *Development* **143**, 880–891.
95 Ferg M, Armant O, Yang L, Dickmeis T, Rastegar S & Strahle U (2014) Gene transcription in the zebrafish embryo: regulators and networks. *Brief Funct Genomics* **13**, 131–143.
96 Wilson AC & Sweeney LB (2023) Spinal cords: symphonies of interneurons across species. *Front Neural Circuits* **17**, 1146449.
97 Brown CR, Butts JC, McCreedy DA & Sakiyama-Elbert SE (2014) Generation of v2a interneurons from mouse embryonic stem cells. *Stem Cells Dev* **23**, 1765–1776.
98 Xue W, Li B, Liu H, Xiao Y, Li B, Ren L, Li H & Shao Z (2023) Generation of dorsoventral human spinal cord organoids via functionalizing composite scaffold for drug testing. *iScience* **26**, 105898.
99 Al Oustah A, Danesin C, Khouri-Farah N, Farreny MA, Escalas N, Cochard P, Glise B & Soula C (2014) Dynamics of sonic hedgehog signaling in the ventral spinal cord are controlled by intrinsic changes in source cells requiring sulfatase 1. *Development* **141**, 1392–1403.
100 Djenoune L, Khabou H, Joubert F, Quan FB, Nunes Figueiredo S, Bodineau L, Del Bene F, Burckle C, Tostivint H & Wyart C (2014) Investigation of spinal cerebrospinal fluid-contacting neurons expressing PKD2L1: evidence for a conserved system from fish to primates. *Front Neuroanat* **8**, 26.
101 Djenoune L, Desban L, Gomez J, Sternberg JR, Prendergast A, Langui D, Quan FB, Marnas H, Auer TO, Rio JP *et al.* (2017) The dual developmental origin of spinal cerebrospinal fluid-contacting neurons gives rise to distinct functional subtypes. *Sci Rep* **7**, 719.
102 Nakamura Y, Kurabe M, Matsumoto M, Sato T, Miyashita S, Hoshina K, Kamiya Y, Tainaka K, Matsuzawa H, Ohno N *et al.* (2023) Cerebrospinal fluid-contacting neuron tracing reveals structural and functional connectivity for locomotion in the mouse spinal cord. *Elife* **12**, e83108.
103 Di Bella DJ, Carcagno AL, Bartolomeu ML, Pardi MB, Lohr H, Siegel N, Hammerschmidt M, Marin-Burgin A & Lanuza GM (2019) Ascl1 balances neuronal versus ependymal fate in the spinal cord central canal. *Cell Rep* **28**, 2264–2274.e3.
104 Parmentier C, Hameury E, Dubessy C, Quan FB, Habert D, Calas A, Vaudry H, Lihrmann I & Tostivint H (2011) Occurrence of two distinct urotensin II-related peptides in zebrafish provides new insight into the evolutionary history of the urotensin II gene family. *Endocrinology* **152**, 2330–2341.
105 Hale ME, Ritter DA & Fetcho JR (2001) A confocal study of spinal interneurons in living larval zebrafish. *J Comp Neurol* **437**, 1–16.
106 Higashijima S, Masino MA, Mandel G & Fetcho JR (2004) Engrailed-1 expression marks a primitive class of inhibitory spinal interneuron. *J Neurosci* **24**, 5827–5839.
107 Higashijima S, Schaefer M & Fetcho JR (2004) Neurotransmitter properties of spinal interneurons in embryonic and larval zebrafish. *J Comp Neurol* **480**, 19–37.
108 Francius C, Harris A, Rucchin V, Hendricks TJ, Stam FJ, Barber M, Kurek D, Grosveld FG, Pierani A, Goulding M *et al.* (2013) Identification of multiple subsets of ventral interneurons and differential distribution along the rostrocaudal axis of the developing spinal cord. *PLoS One* **8**, e70325.
109 Briscoe J & Ericson J (1999) The specification of neuronal identity by graded Sonic Hedgehog signalling. *Semin Cell Dev Biol* **10**, 353–362.
110 Zhang H, Shevtsova NA, Deska-Gauthier D, Mackay C, Dougherty KJ, Danner SM, Zhang Y & Rybak IA (2022) The role of V3 neurons in speed-dependent interlimb coordination during locomotion in mice. *Elife* **11**, e73424.
111 Goulding M, Lanuza G, Sapir T & Narayan S (2002) The formation of sensorimotor circuits. *Curr Opin Neurobiol* **12**, 508–515.
112 Jessell TM (2000) Neuronal specification in the spinal cord: inductive signals and transcriptional codes. *Nat Rev Genet* **1**, 20–29.
113 Kuscha V, Barreiro-Iglesias A, Becker CG & Becker T (2012) Plasticity of tyrosine hydroxylase and serotonergic systems in the regenerating spinal cord of adult zebrafish. *J Comp Neurol* **520**, 933–951.

114 Ballion B, Branchereau P, Chapron J & Viala D (2002) Ontogeny of descending serotonergic innervation and evidence for intraspinal 5-HT neurons in the mouse spinal cord. *Brain Res Dev Brain Res* **137**, 81–88.
115 Newton BW & Hamill RW (1988) The morphology and distribution of rat serotoninergic intraspinal neurons: an immunohistochemical study. *Brain Res Bull* **20**, 349–360.
116 Cazalets JR, Sqalli-Houssaini Y & Clarac F (1992) Activation of the central pattern generators for locomotion by serotonin and excitatory amino acids in neonatal rat. *J Physiol* **455**, 187–204.
117 Craven SE, Lim KC, Ye W, Engel JD, de Sauvage F & Rosenthal A (2004) Gata2 specifies serotonergic neurons downstream of sonic hedgehog. *Development* **131**, 1165–1173.
118 Deneris E & Gaspar P (2018) Serotonin neuron development: shaping molecular and structural identities. *Wiley Interdiscip Rev Dev Biol* **7**, doi: 10.1002/wdev.301
119 Nishimaru H & Kudo N (2000) Formation of the central pattern generator for locomotion in the rat and mouse. *Brain Res Bull* **53**, 661–669.
120 Peng CY, Yajima H, Burns CE, Zon LI, Sisodia SS, Pfaff SL & Sharma K (2007) Notch and MAML signaling drives Scl-dependent interneuron diversity in the spinal cord. *Neuron* **53**, 813–827.
121 Crone SA, Quinlan KA, Zagoraiou L, Droho S, Restrepo CE, Lundfald L, Endo T, Setlak J, Jessell TM, Kiehn O *et al.* (2008) Genetic ablation of V2a ipsilateral interneurons disrupts left-right locomotor coordination in mammalian spinal cord. *Neuron* **60**, 70–83.
122 Azim E, Jiang J, Alstermark B & Jessell TM (2014) Skilled reaching relies on a V2a propriospinal internal copy circuit. *Nature* **508**, 357–363.
123 Kang K, Lee D, Hong S, Park SG & Song MR (2013) The E3 ligase mind bomb-1 (Mib1) modulates Delta-Notch signaling to control neurogenesis and gliogenesis in the developing spinal cord. *J Biol Chem* **288**, 2580–2592.
124 Britz O, Zhang J, Grossmann KS, Dyck J, Kim JC, Dymecki S, Gosgnach S & Goulding M (2015) A genetically defined asymmetry underlies the inhibitory control of flexor-extensor locomotor movements. *Elife* **4**, e04718.
125 Zhang J, Lanuza GM, Britz O, Wang Z, Siembab VC, Zhang Y, Velasquez T, Alvarez FJ, 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.