Evolution of vertebrate spinal cord patterning

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Key findings:

Retinoic acid patterns anterior-posterior axis in chordates through *Hox*.

Dorsal-ventral patterning is more elaborate in vertebrates, which might be a reflection of two rounds of whole genome duplication.

Medial ventricular progenitor zone is a vertebrate innovation.

Abstract

The vertebrate spinal cord is organised across three developmental axes, anterior-posterior (AP), dorsal-ventral (DV) and medial-lateral (ML). Patterning of these axes is regulated by canonical intercellular signalling pathways: the AP axis by Wnt, FGF and Retinoic Acid (RA), the DV axis by Hedgehog, Tgfβ and Wnt, and the ML axis where proliferation is controlled by Notch. Developmental time plays an important role in which signal does what, when. Patterning across the three axes is not independent, but linked by interactions between signalling pathway components and their transcriptional targets. Combined this builds a sophisticated organ with many different types of cell in specific AP, DV and ML positions.

Two living lineages share phylum Chordata with vertebrates, amphioxus and tunicates, while the jawless fish such as lampreys, survive as the most basally divergent vertebrate lineage. Genes and mechanisms shared between lampreys and other vertebrates tell us what predated vertebrates, while those also shared with other chordates tell us what evolved early in chordate evolution. Between these lie vertebrate innovations: genetic and developmental changes linked to evolution of new morphology. These include gene duplications, differences in how signals are received, and new regulatory connections between signalling pathways and their target genes.

1. The evolution of the vertebrate nervous system

Vertebrates develop complex nervous systems, with a level of sophistication that far exceeds that of invertebrates. The only challengers to this claim might be cephalopod molluscs, and some of the larger arthropods. Many of the complexities shown by vertebrates are considered innovations (Shimeld and Holland, 2000; Holland et al., 2013), and evolutionary biologists have long been interested in when and how vertebrate neural complexity evolved. Most studies on this topic have focused on the brain or the neural crest. This is not surprising, since the brain is the centre of characteristics such as upper level neural processing and memory, while the neural crest has long been recognised as a pivotally-important tissue in vertebrate evolution, giving rise to many structures considered to be vertebrate innovations (Gans and Northcutt, 1983; Northcutt and Gans, 1983). There is, however, more to the vertebrate nervous system than the brain and neural crest. Nervous systems are typically divided in to central (CNS) and peripheral (PNS) components. The vertebrate PNS derives primarily form neural crest cells, with cranial neurogenic placodes also playing a role. The evolution of neural crest and placodes has been extensively reviewed elsewhere (Patthey et al., 2014; Schlosser et al., 2014; Green et al., 2015) and will not be further considered here. The CNS is often dichotomised into the brain and spinal cord. While this makes sense from anatomical and functional perspectives, whether the separation is justified on developmental and evolutionary grounds is much more debatable. Aspects of spinal cord organisation and development extend into the hindbrain, including patterning by Hox genes, suggesting the separation may be better thought of as into a posterior, Hox-expressing territory (hindbrain and spinal cord) and anterior Hox-free territory (midbrain and forebrain). Recent work also shows deep and fundamental differences may exist in the gene regulatory networks (GRNs) that regulate the formation of the anterior and posterior neural cells very early in development (Metzis et al., 2018), and some authors have even speculated there may be separate evolutionary origins for anterior and posterior CNS

components, traceable to separate anterior and posterior nervous systems in an early ancestor of the majority of extant animals (Arendt et al., 2016).

There is clearly still much to learn about the similarities and differences between anterior and posterior CNS in vertebrates and other animal groups, but regardless of the outcome the spinal cord has received relatively little attention from evolutionary biologists. This is in stark contrast to developmental biologists, who have dissected spinal cord development with impressive detail. This reflects a combination of its relative simplicity and its experimental tractability for gene manipulation and imaging. It has resulted in the spinal cord becoming something of a 'model organ system' for understanding core developmental processes such as morphogen patterning and the balance between proliferation and differentiation (Gouti et al., 2015). This depth of knowledge represents an opportunity to explore the relationship between genetic change at the gene and genome level, developmental processes, and phenotypic consequences in terms of nervous system complexity. Key phenotypic changes in this regard might include increased cell number, new cell types, innovation in cell patterning, and mechanisms for axon navigation. In this review we will first summarise how vertebrate spinal cords develop. We will then relate this to genetic change, including gene duplication, with comparison to early diverging vertebrates such as lampreys, and to the vertebrates' nearest invertebrate relatives, tunicates and amphioxus (Figure 1).

2. Spinal cord developmental anatomy

The adult spinal cord of vertebrates serves a number of functions. Most obviously it relays signals between the periphery and the brain. Put simplistically, sensory information is received via the dorsal nerve roots and passed on to the brain, while motor instructions are passed from the brain to the motor neurons which connect to the muscles. The spinal cord may also manage reflex actions like escape or withdraw responses, and house central pattern generators managing rhythmic motor activity. Many different types of cell are needed

to perform these functions, including various interneurons. While there are some important differences in spinal cords between vertebrate groups, for example in motor control circuits in limbed versus finned vertebrates, cell types and their distributions appear fundamentally conserved (Goulding, 2009).

The developmental origins of these different cell types has been intensively studied in model systems, especially in chick and mouse. Across the Dorsal-Ventral (DV) axis the embryonic spinal cord is divided into multiple discrete zones, each giving rise to a defined suite of cell types. Proliferative progenitor cells are sited ventricularly (by the lumen of the spinal cord) in stem cell zones that produce new cells through an extended period of development. Cells born from the same progenitor cell zone at different developmental times may have different fates, leading to Medio-Lateral (ML) diversity of cell types at the same DV level. The spinal cord is also patterned along the Anterior-Posterior (AP) axis. Before considering the evolution of these axes, we will first summarise the molecular control of their development as understood from model vertebrates.

3. Patterning the vertebrate spinal cord: the AP axis

The spinal cord grows from anterior to posterior, in common with other axial tissues such as the somitic and intermediate mesoderm. New cells are born in the posterior growth zone, the tailbud, and as these cells continue to proliferate the tailbud grows posteriorly, leaving some cells behind in the forming spinal cord (Akai et al., 2005). As developmental time passes, these cells become progressively distant from the tailbud and may start to differentiate. AP identity in spinal cord cells is regulated by Hox gene expression. Establishment and maintenance of Hox gene expression in the spinal cord is a complex affair, involving regulation by multiple external signals, by interactions between Hox genes, and by change in chromatin state through developmental time. There is insufficient space here to detail all of these, and the reader is referred to a recent review for a detailed exploration of these issues

(Deschamps and Duboule, 2017). However, some key generalisations can be extracted (Figure 2).

Wnt signalling plays a role in posterior specification in many bilaterians (Niehrs, 2010), and in vertebrates is involved in early regulation of the Hox complexes (Deschamps and Duboule, 2017). It has also long been known from cell culture, explant and signalling pathway manipulation studies that Hox genes differ in their sensitivity to signalling molecules like Retinoic Acid (RA) and Fibroblast Growth Factor (FGF). RA is secreted from newly-formed somites, while FGF (and other signalling molecules like the Tgfβ family member Gdf11) are produced by the tailbud (Liu et al., 2001; Diez del Corral et al., 2003). Since both tailbud and somitogenesis progress synchronously from anterior to posterior, all spinal cord cells should in principle move through developmental time from a high FGF, to a high RA environment. Exactly when and how these signals regulate Hox expression though has been tricky to decipher. Under RA treatment in chick embryos, the anterior boundaries of neural tube expression *Hoxb1, Hoxb3, Hoxb4* and *Hoxb5* extend, but their expression is not affected by FGF treatment; conversely, FGF treatment anteriorises the expression of *Hoxb6, Hoxb7, Hoxb8* and *Hoxb9,* but these are not sensitive to RA in the same assay (Bel-Vialar et al., 2002). However, others showed that *Hoxc6* and *Hoxc9* expression in explants respond to both RA and FGF treatment, with *Hoxc6* expression increasing when treated with RA and reducing when treated with FGF, whereas *Hoxc9* expression responded reciprocally (Liu et al., 2001).

Such differences in observation could be explained by studies using different methods and systems (for example whole embryo in vivo, versus explants in vitro), but developmental time also plays a critical role. Tailbud derived FGF regulates proliferation (Diez del Corral et al., 2003; Diez Del Corral and Morales, 2017), but cells that spend more time in the tailbud appear to show progressive opening of chromatin across Hox clusters (Soshnikova and

Duboule, 2009). How exactly this works needs more study, but it suggests a model in which developmental time regulates Hox cluster chromatin state, which is then acted on by other signals. Anterior spinal cord cells derive from progenitors that have fewer Hox genes accessible for transcription, while more posterior cells derive from progenitors that have more Hox genes accessible. Accessible Hox genes might then be regulated by signals like RA, and FGF. The homeobox gene *Cdx* is a good candidate for mediating FGF/Gdf11 regulation of Hox (Gaunt, 2017; Neijts et al., 2017), and RA probably directly regulates many Hox genes through RA responsive elements (RAREs) identified in Hox gene enhancers (Krumlauf, 2016). In addition some studies suggest the Hox genes may cross regulate, for example *Hoxc6* and *Hoxc9* appear to cross repress each other and hence establish the boundary between the thoracic and lumbar regions of the spinal cord (Dasen et al., 2003).

4. Patterning the vertebrate spinal cord: the DV axis

Spinal cord DV patterning has become a model for understanding how morphogen signals may pattern a field of cells, with experimental approaches and mathematical models integrating signalling, downstream GRN interactions and developmental time. For a detailed exploration of this the reader is referred to recent reviews (Briscoe and Small, 2015; Gouti et al., 2015). Here, we will draw out the key points, focusing on aspects that allow comparison to other animals and hence evolutionary insight (Figure 2).

The spinal cord of amniote embryos is organised into a series of DV progenitor (or stem cell) zones (Figure 3). Ventrally these are named p0-p3, and pMN, with the first four producing interneurons and pMN producing motor neurons of various sorts, plus some other cells. As a generality, these cells are involved in controlling motor output (reviewed by (Goulding, 2009). Dorsally are dP1-6, which primarily produce interneurons involved in managing sensory input from the PNS. Many of the target subpopulations of interneuron innervation are known, as are the neurotransmitters they express. The expression of numerous transcription factor

genes have also been mapped across these progenitor zones and the differentiated cells they produce. There is insufficient space to document this here: key aspects are shown in Figure 3, and the reader is referred to recent reviews for more detail (Goulding, 2009; Alaynick et al., 2011; Lai et al., 2016).

The ventral spinal cord is dependent on the morphogen Shh secreted from the floorplate and notochord, evidenced by failure of ventral spinal cord patterning in *Shh*$^{-/-}$ mice (Chiang et al., 1996). Other factors may also have contributions to ventral patterning including Eph/Ephrin signalling (Laussu et al., 2017), and *Chordin*, which is expressed in the notochord and inhibits Bmp signalling (Sasai et al., 1994), but Hedgehog (Hh) signalling mediated by Shh has a dominant role. Pairs of cross repressive transcription factor genes lie downstream of the Shh signal. For example: *Nkx2.2* and *Olig2* cross repress to establish the boundary between the p3 and pMN zones; *Nkx6.1* and *Dbx2* cross repress to establish the boundary between p2 and p1; *Nkx6.2* and *Dbx1* cross repress to establish the boundary between p1 and p0 (Briscoe et al., 2000; Sun et al., 2003); *Irx3* and *Olig2* cross-repress to form p2/pMN boundary (Novitch et al., 2001); *Sp8* and *Pax6* class I progenitor zone markers cross-repress *Nkx2.2* class II p3 zone marker to form pMN/p3 boundary (Li et al., 2014b). While these mechanisms are not fully resolved, it would appear these transcription factors generally act by interacting with Groucho/TLE repressors, and that as well as repressing each other they also repress a broad swathe of other genes that are normally expressed in other DV compartments (Kutejova et al., 2016). Receipt of Shh signalling is presumed to be quantitative, mediated by a balance between active and repressive forms of Gli proteins. To add additional complexity, the three Gli proteins involved (Gli1, Gli2 and Gli3) have different abilities to produce active and repressive forms, and their genes are also differently regulated by Hh signalling. All this creates a dynamic regulatory system that mathematical modelling suggests has interesting properties, including bi-stable outputs and the ability to scale across differently-sized fields of cells (Briscoe and Small, 2015; Uygur et al., 2016).

Views on patterning of the dorsal spinal cord are more controversial. The roof plate produces multiple signalling molecules including Wnt1 and Wnt3, and Tgfβ members including Bmp2, Bmp4, Bmp7 and Gdf7 (Figure 2). Tozer and colleagues (Tozer et al., 2013) proposed that the gradient and exposure time of BMPs from the roof plate is responsible for patterning dorsal spinal cord cells. However others have argued (Andrews et al., 2017) that the nature of the BMPs (rather than just their concentration) is responsible for cell type specification. BMP4 upregulates pSmad1/5/8 more efficiently than BMP7, and ectopic expression of *Bmp4* increase the dI1 and dI2 populations while ectopic *Bmp7* and *Gdf7* increases roof plate, dI1 and dI3 populations. In addition to Tgfβ signals, Wnt signalling is also involved in dorsal spinal cord patterning. The *Wnt1/Wnt3a* double homozygous mutant shows loss of expression of dI1 and dI2 markers *LH2, Islet1, Math1* and *Ngn1,* and gain of expression of dI3 markers *Pax2, Lim1/2* and *Mash1* in the dorsal spinal cord, while having no effect on roof plate expression of *Bmp4, Gdf7* and *Msx1* (Muroyama et al., 2002). This suggests a role for Wnt signalling in dorsal patterning, though Wnt also affects cell proliferation (see below). In summary, while there are clearly roles for both Tgfβ and Wnt signalling in development of the dorsal spinal cord progenitor cell zones, exactly which signal does what and how, how they inter-relate, and how this connects to proliferation, needs much more study.

To make things even more complex, Wnt signalling also induces the expression of *Gli3,* which generally represses targets of Shh signalling (Alvarez-Medina et al., 2008; Yu et al., 2008). Thus dorsal and ventral signals are not independent entities, but potentially interact within receiving cells. Furthermore AP and DV patterning are also interlinked. Posterior signals may block DV patterning from commencing: for example FGF regulates the expression of *Nkx1.2* in posterior cells, and this prevents cells from responding to Shh signals until *Nkx1.2* levels have dropped sufficiently (Sasai et al., 2014). RA also upregulates some progenitor cell zone genes (Novitch et al., 2003), and in vitro study shows that *Shh*

expression depends on RA concentration (Okada et al., 2004). These interactions coordinate the progression from a relatively naïve tailbud progenitor, to an AP- and DV-specified progenitor, and eventually on to a differentiated cell.

5. Patterning the vertebrate spinal cord: the ML axis, progenitor cell maintenance, and differentiation

As AP and DV patterning progress, the spinal cord develops in an 'inside to outside' manner: undifferentiated proliferative cells lie ventricularly, forming progenitor cell zones with DV pattern as discussed above (Figure 3). When these cells divide, one or both daughter cells may migrate laterally to differentiate. Cells born later in development migrate through fields of earlier-differentiated cells to differentiate, meaning cells born in the same progenitor cell zone but at different times may migrate into different environments and hence adopt different fates. In this way multiple cell types can form over time from the same progenitor cell population. The best studied example of this is the pMN zone, where motor neurons of different types, then later oligodendrocytes, are successively born from the same progenitor cell zone (Sockanathan et al., 2003).

Expression of SoxB1 genes marks neural stem cells, and the SoxB1 gene *Sox2* is sufficient to maintain progenitor cells at the ventricular zone (Graham et al., 2003). *Foxp2* and *Foxp4* are required and sufficient for neural differentiation, opposing SoxB1 activity, and with *Foxp*-expressing cells repressing the expression of junction protein genes such as *N-cadherin*, detaching from neuroepithelium and migrating laterally (Rousso et al., 2012). Notch signalling controls which cells undertake this transition, with sustained Notch signalling needed to maintain progenitor cells in the progenitor zones (Wettstein et al., 1997; Appel et al., 2001). From a cell-to-cell interaction point of view, Isomura and colleagues have shown through single-cell bioluminescence imaging of progenitors that Notch-Dll signalling can entrain an intracellular *Hes1* oscillating negative feedback loop (Isomura et al., 2017). It

would be interesting to know what breaks the feedback loop to direct the cell to differentiation.

What regulates Notch signalling, the continued maintenance of progenitor cells, and the balance between rates of proliferation and differentiation is not well understood but many factors have been implicated, including several of the signals that pattern AP and DV axes. As discussed above, FGF maintains proliferative progenitors in the tailbud (Mathis et al., 2001), possibly via Notch signalling (Akai et al., 2005), while RA promotes differentiation (Diez del Corral et al., 2003). Shh signalling in the ventral neural tube is sufficient and required for *Jag2* expression, a Notch ligand. Jag2 activity prevents premature differentiation of oligodendrocytes and inhibits differentiation of motor neuron progenitors (Rabadan et al., 2012). Notch signalling may also impact on the receipt of Shh signalling, via effects of the localisation of the Hh receptor Ptc in the primary cilium (Kong et al., 2015).

Bmp6 also speeds up cell cycle transition (Andrews et al., 2017; Le Dreau et al. 2014), indicating Bmp may also be involved in ML development (Le Dreau et al., 2014; Andrews et al., 2017). Furthermore Shh and Wnt signalling control different stages of the cell cycle through regulation of cyclin D1, E, A, B, with Shh being epistatic to Tcf3/4 (a Wnt-regulated transcription factor) when regulating cyclin D1 (Alvarez-Medina et al., 2008). Cell cycle regulation in neural tube development is more extensively reviewed elsewhere (Molina and Pituello, 2017; Saade et al., 2018). In addition another ventral signal, Sema3B, may also regulate progenitor maintenance (Arbeille et al., 2015).

While still not fully pieced together, these data suggest regulation of proliferation is complex, involves inputs from both AP and DV signalling pathways, and may vary along both AP and DV axes as well as through developmental time at the same axial location. As a generalisation though, jawed vertebrates use these mechanisms to maintain a population of

ventricular progenitors which continue to divide and produce new differentiating cells. This allows embryos to make more cells and different types of cells, both components of a complex CNS. It may also allow them to set progenitor cell populations aside with the potential for these to persist into the adult and engage in the repair of damaged spinal cords (reviewed by (Grandel and Brand, 2013)).

6. Outgroups and evolutionary origins: spinal cords, cell types and development in other chordates

Amongst living animals, cyclostomes (lampreys and hagfishes) represent the earliest diverging lineage of vertebrates. Two other living lineages share phylum Chordata with the vertebrates: cephalochordates (amphioxus) and tunicates (including sea squirts). Their evolutionary relationships are shown in Figure 1, and they offer a window into the timing and genetic basis of evolutionary change in spinal cord patterning.

Lampreys are moderately well studied and in many respects similar to jawed vertebrates, but with important differences. For example they appear to have a different history of gene and genome duplication, and lack myelination and hence are assumed to have differences in glial cell development (Smith et al., 2013; Smith et al., 2018). The hindbrain is segmented into rhombomeres with many similarities to those of jawed vertebrates, including patterns of Hox expression and neural crest organisation (Parker et al., 2014; Parker et al., 2016; Parker et al., 2019). Adult spinal cords are quite well studied as a model for understanding reflex circuitry and regeneration (Shifamn and Selzer, 2015; Herman et al., 2018), and found to have a variety of identified cell types (Figure 4: discussed more below). The sea squirt *Ciona* is very well studied, but like others in its lineage the spinal cord region of its CNS is secondarily reduced and without neurons or a tailbud growth zone (Figure 1). This derived status limits its use in understanding ancestral spinal cord patterning. Amphioxus has been quite well studied and offers significant insight into the pre-vertebrate condition. Limitations

are that most studies have focused on embryos with adult spinal cord cell types not well described, and that most embryogenesis studies focus on the anterior CNS, and it is questionable whether this region is comparable to the spinal cord, or instead homologous to the hindbrain.

6.1 Nerve cell types in adult lamprey and amphioxus spinal cords

Traditionally, the function of neurons are inferred from the type of neurotransmitters they secrete (Table 1), with glutamatergic rhythm-generating neurons signalling to acetylcholinergic motor neurons, and to glycinergic commissural neurons to inhibit motor neurons on the contralateral side (Goulding, 2009; Kiehn, 2016). However, recent studies have shown co-expression of two neurotransmitters in a single neuron (Vaaga et al., 2014), with a comprehensive zebrafish study (Pedroni and Ampatzis, 2019) identifying a small population of neurons with three neurotransmitters, acetylcholine, GABA and glutamate. Hence while neurotransmitter distributions can help illustrate the number of different cell types, relying on them alone as an indication of cell homology may be problematic.

Figure 4 shows schematic cross sections of spinal cords of amphioxus and lamprey, compared to a zebrafish. Lampreys show similarity to other vertebrates (Figure 4 and references therein), with a diversity of cell types as assessed by neurotransmitter complements. There are few data on neurotransmitter distributions in adult amphioxus, limited studies showing GABAergic (Anadon et al., 1998), dopaminergic and serotonergic (Candiani et al., 2001; Moret et al., 2004) neuron distributions. Other studies have characterised amphioxus adult neurons by their immunoreactivity to a variety of neuropeptides including arginine vasopressin, oxytocin, angiotensin II, cholecystokinin, urotensin, natriuretic peptide, FMRFamide, neuropeptide Y, calretinin and acetylated tubulin (Uemura et al., 1994; Pestarino and Lucaroni, 1996; Castro et al., 2003; Castro et al., 2004; Castro et al., 2015). However these are not that informative for comparison across taxa as similar data from other species are not well developed. There are more data on embryonic

and larval amphioxus (Candiani et al., 2012), showing that glutamatergic, serotoninergic, GABAergic, glycinergic and acetylcholinergic neurons can all be identified in the anterior CNS. However none were identified more posteriorly. As considered more below, it is the posterior region that may be directly comparable to the spinal cord of vertebrates, with anterior regions potentially homologous to fore/mid brain and hindbrain. A possible explanation for the absence of neurotransmitter positive cells in posterior regions is that posterior cells had yet to differentiate and take on an identity. Irrespective, it means caution is needed in comparison between current understanding of neurotransmitter distributions, and more work is needed on the posterior larval and adult CNS of amphioxus.

6.2 AP spinal cord patterning in lampreys, sea squirts and amphioxus

Mechanisms of AP spinal cord patterning have not been well studied in lamprey embryos, although what is known is consistent with a general similarity to jawed vertebrates. Embryos grow from anterior to posterior via a tailbud, forming new somites and spinal cord over more than two weeks of development. RA appears to be synthesised by somite cells (Castillo et al., 2010) and Hox gene expression has been identified in the hindbrain and spinal cord, though posterior Hox genes have not been well studied (Parker et al., 2014; Pascual-Anaya et al., 2018; Parker et al., 2019).

Some *Ciona* Hox genes are expressed in the CNS, but the *ascidian* Hox cluster is fragmented (Ikuta et al., 2004; Sekigami et al., 2017) and this is not an ancestral state. Manipulation of RA and FGF signalling in *Ciona* can affect AP patterning, including Hox expression (Katsuyama et al., 1995; Pasini et al., 2012). Amphioxus Hox genes are expressed in a nested pattern in the CNS, and in the genome are located in a single canonical cluster (Garcia-Fernandez and Holland, 1994; Wada et al., 1999). There is anteriorisation of *Hox1*, *Hox2*, *Hox3*, *Hox4* and *Hox6* expression in the neural tube when embryos are treated with RA. *Hox14* is also responsive to RA even though it is only

expressed in the posterior notochord, gut and cerebral vesicle at larval stage (Schubert et al., 2006; Pascual-Anaya et al., 2012). Several Wnt genes show tailbud expression (Schubert et al., 2001; Somorjai et al., 2018), though only limited manipulation of Wnt signalling has been conducted in amphioxus to date (Dailey et al., 2017) and we do not know whether Wnt regulates Hox. FGF inhibition alone does not seem to have an effect on *Hox1* expression but when coupled with an RA antagonist there is no *Hox1* expression in the neural plate (Bertrand et al., 2015). This study also found no evidence for opposing effects of RA and FGF on AP patterning in amphioxus, since inhibition of FGF caused loss of expression of typical downstream genes *ER81*, *Erm*, *Pea3* and *Sprouty*, but these remained unchanged when RA was inhibited. Similarly RA inhibition did not affect *Cdx* and *Xlox*. In the larval stage of amphioxus, GABA-immunoreactive neurons exists in clusters along the neural tube posterior to the cerebral vesicle boundary. Exposure to BMS493, an RA antagonist, has a posteriorising effect on these cells with the gain of an extra cluster, while RA treatment has an anteriorising effect, with three GABA clusters lost (Zieger et al., 2018). *Tlx* and *Prdm12* seem to have anteriorised expression in the neural tube under a high concentration of RA. When RA signalling is inhibited, their neural tube expression is lost or reduced (Carvalho et al., 2017).

A consideration when comparing the outcomes of these experiments to studies of vertebrates, including lampreys, is whether homologous regions of the CNS are being compared, particularly with respect to the hindbrain and spinal cord. The primary pigment spot of amphioxus develops at about the level of the boundary between somite 4 and somite 5, and many studies focus on cells anterior to this point. However this region is within the domain of *Hox1* and *Hox3* expression, with the anterior boundary of *Hox4* mapping around the level of somite 6 (Wada et al., 1999). If we consider Hox gene expression boundaries as landmarks between lineages, this region would be homologous to the vertebrate hindbrain and not the spinal cord. Spinal cord would be homologous to regions posterior to about

somite 6/7. Hence some comparisons between vertebrate spinal cord and amphioxus CNS have to be made cautiously, acknowledging the lack of certainty around what exactly is, and is not, homologous.

6.3 DV spinal cord patterning in lampreys, sea squirts and amphioxus

Lamprey, amphioxus and sea squirt embryos all show DV polarisation of the spinal cord regions of their CNS. In most lineages orthologous signalling molecules are also expressed in similar positions as in jawed vertebrates. In lamprey the spinal cord is exposed to dorsal Bmp and Wnt signalling (McCauley and Bronner-Fraser, 2004; Guerin et al., 2009), with Hh localised to notochord and floor plate (Kano et al., 2010; Sugahara et al., 2011). Hh is also expressed by notochord cells in *Ciona* (Takatori et al., 2002), and by notochord and floor plate cells in amphioxus (Shimeld, 1999), while both Bmp and Wnt gene expression in amphioxus is consistent with a role in dorsal patterning (Panopoulou et al., 1998; Hu et al., 2017). The spinal cord is thus bounded across the DV axis by the expression of the same signals in all chordates.

Lamprey homologues of some spinal cord DV patterning genes have also been studied. The Hh receptor, *Patched* is expressed in the ventral-medial neural tube (Hammond et al., 2009) and a Gli gene that is probably orthologous to *Gli3* is expressed in the dorsal neural tube (Sugahara et al., 2011). These data imply lamprey spinal cord is patterned by Hh signalling, though this has yet to be validated experimentally. Hammond et al. (2009) also showed *Engrailed*, a V1 neuron marker in jawed vertebrates, is expressed in the ventral medial neural tube, consistent with the presence of a homologous cell population. *Pax3/7*, a dI2-6 marker, is expressed in the dorsal neural tube (Kusakabe and Kuratani, 2005), while *OligA* is expressed in dorsal and ventral spinal cord (Lara-Ramirez et al., 2019), paralleling jawed vertebrate Olig gene expression in pMN and dI1-3 (Alaynick et al., 2011). An insightful recent study confirms that *Pax6* and *Nkx2.2* are expressed in the spinal cord, and in addition

showed a specific cell population in the ventral spinal cord expresses *Hb9*, a MN marker from the Mnx homeobox gene family (Yuan et al., 2018). Although the spinal cord region has been overlooked in many other studies, hindbrain expression of other neural tube patterning genes has been demonstrated, including *Msx, Dbx, Wnt5, Dlx, Gsh,* and *Isl*, and these are likely to extend into the spinal cord (Shigetani et al., 2002; Guerin et al., 2009; Cerny et al., 2010; Sugahara et al., 2011). These data combined suggest many aspects of DV spinal cord patterning are conserved between lampreys and jawed vertebrates.

As considered above, amphioxus has a much simpler CNS than any vertebrate, with fewer cells and probably also fewer types of cells. ML progenitor zones are lacking (see section 6.4), with evidence instead suggesting that individual cell types differentiate in situ across the DV axis. Nevertheless, studies have shown similarities in gene expression patterns to vertebrates, the most comprehensive being a study focused on midneurula stage (Albuixech-Crespo et al., 2017). This showed that:

- *Hh, Nkx2.1, Nkx6* and *Gsc* are expressed by the floorplate.
- *Six3/6*, *Lhx2/9b*, *Zic*, *Msx*, *Pax2/5/8*, and *Pax3/7* are expressed in the peripheral neural plate, which will become the dorsal neural tube as the plate folds.
- *Pou3f*, *Sim*, *FoxD*, *Meis*, *Lef*, *Lhx1/5*, *Hox3*, *Hox6*, *FoxB* are expressed in the internal neural plate, which will be the ventral neural tube.
- O*tx*, *Gbx*, *Fezf*, *Irx*, *Pax4/6*, *Six3/6*, *Nkx2.2*, *Meis*, *Rx*, *Hox1*, *Wnt3*, *Wnt7*, *Nova*, *Ebf/ COE* are expressed in both internal and peripheral neural plate.

Broadly speaking, these patterns correspond to the relative expression of their orthologues in jawed vertebrates (though there are also many differences). However the focus on the midneurula stage also means the neural plate at this time may be homologous to the brain only, as discussed above. At later developmental stages many of these genes mark scattered, isolated cells in the neural tube including *Islet* (Jackman and Kimmel, 2002), *Pax3/7* (Holland et al., 1999), *Evx* (Ferrier et al., 2001b), *OligA* (Beaster-Jones et al., 2008),

prdm12, engrailed (Thélie et al., 2015), *Tlx* (Kaltenbach et al., 2009), *Msx* (Sharman et al., 1999), *Mnx/Hb9* (Ferrier et al., 2001a), and *Err* (Schubert et al., 2006), with the exception of *IrxA* being expressed along the whole neural tube at late neurula stage (Beaster-Jones et al., 2008).

To date there are insufficient functional studies for us to determine if and how Hh, Bmp and Wnt signalling might be involved in DV patterning of the amphioxus spinal cord. Wnt signalling has yet to be studied at the right developmental stages. Bmp signalling is known to regulate the patterning of epidermal neurons, though its impact on the spinal cord is not clear (Lu et al., 2012). Hh signalling has been studied with the smoothened inhibitor cyclopamine and no changes in neural patterning gene expression were observed (Ono et al., 2018). However, the study focus was gill slit formation, and the drug treatment time only a 20 minute window, which might explain this outcome. Through generation of $Hh^{-/-}$ embryos using TALEN knockout, the role of Hh signalling in regulating left right asymmetry in amphioxus has also been studied (Hu et al., 2017). Again, neural patterning was not the focus of the work so few relevant genes or developmental stages were analysed. However the study did show that *Err* expression became symmetrical in the brain and was lost from more posterior neural tube at the single stage shown. This hints at a role for Hh signalling in amphioxus neural patterning, though this needs more focused investigation with appropriate marker genes.

6.4 ML spinal cord patterning in lampreys, sea squirts and amphioxus: differences in cell number

As considered in Section 5, jawed vertebrate embryos build complexity into spinal cords via a ventricular progenitor cell population that produces new cells over a relatively long period of development. This yields spinal cords with multiple cell layers, and gives the potential for different cell types to be born at different times. Do other chordates operate in similar

mechanisms? Lamprey embryos do, and resemble jawed vertebrates with a well-defined progenitor cell zone visible both by cell morphology and gene expression in brain and spinal cord (Guerin et al., 2009; Lara-Ramirez et al., 2019). The mechanism is conserved, since Notch signalling maintains progenitor cells at the ventricular zone, with inhibition of Notch by DAPT causing loss of progenitor cell markers *PCNA, OligA* and *HesB*, and premature differentiation as seen by gain of the differentiation markers *CoeA* and *CoeB,* (Lara-Ramirez et al., 2019).

With their highly reduced posterior CNS, sea squirt embryos do not have cells comparable to the ventricular progenitors of jawed vertebrates. Amphioxus also lacks a ventricular progenitor cell zone. Studies of cell proliferation have failed to identify more than a few scattered dividing cells (Bertrand and Escriva, 2011), while Notch signalling is lost early form the neural plate (Holland et al., 2001; Rasmussen et al., 2007) and DAPT inhibition of Notch signalling has no clear effect on the CNS (Lu et al., 2012). This does not mean amphioxus has no proliferation in the CNS. Tailbud cells continue to produce new cells, scattered dividing cells in more anterior regions of the CNS show not all these cells are post-mitotic, and the anterior brain does eventually develop more of a layered structure. However a ventricular proliferative layer is missing throughout the embryonic and larval spinal cord, and this matches the anatomy of the adult spinal cord which, judging from histological cross-sections, remains one to two cells thick from embryo to adult (Bone, 1959; Bone, 1960; Bocina and Saraga-Babic, 2006; Candiani et al., 2012).

6.5 Conservation and innovation in chordate spinal patterning

Overall, the picture that emerges from these studies is that broad organisation of the neural plate shows conserved aspects between amphioxus and vertebrates. This includes AP patterning by Hox and DV organisation, as might be expected from evidence for more ancient similarities in basic nervous system development amongst bilaterally-symmetrical

animals (Holland et al., 2013). Markers of differentiated vertebrate cell types also sometimes show expression in subsets of amphioxus neurons. These may reflect conserved cell types, perhaps also functionally related and expressing similar neurotransmitters as discussed above, but this has not been experimentally demonstrated. The role of patterning signals is much less clear and in need of better experimental validation, especially those involved in the DV axis. Irrespective of the outcome of such work though, it is clear is that amphioxus does not develop ventricular progenitor cell layer as seen in vertebrate. Cells here are not divided into DV zones, rather cells are patterned at the level of individual differentiating cells.

7. A role for gene duplication in spinal cord evolution

It has long been thought that early vertebrate evolution was marked by extensive gene duplication, with broad comparisons of syntenic and paralogous gene organisation between jawed vertebrate and amphioxus genomes seen by most authors as showing this had occurred through two rounds of genome duplication, the '2R' hypothesis (Putnam et al., 2008). New genes offer the potential for new function (neofunctionalisation), and can diverge through splitting ancestral functions between duplicates (subfunctionalisation) or having duplicates maintaining broad ancestral functions with at least one becoming restricted to a more distinct function (specialisation) (Table2). How 2R gene duplication relates to evolutionary change in development and morphology has been hard to pin down, however, since both happened so long ago and intermediate lineages have gone extinct, meaning relating the timings of duplication, potential neofunctionalisation/subfunctionalisation/specialisation and morphology is tricky. Recent observation suggest that specialisation occurs mostly in neural tissue, through gain in regulatory elements producing more complex gene regulatory networks (Marletaz et al., 2018).

Amphioxus and tunicates predate 2R and tend to have single copies of transcription factor family genes, expect where secondary loss has occurred (Putnam et al., 2008; Paps et al., 2012). The position of lampreys with respect to 2R has also been debated. Lamprey genes often fail to resolve well in molecular phylogenetic analyses (Kuraku et al., 2009), and some have suggested additional and/or independent duplications in the lamprey lineage (e.g. (Escriva et al., 2002; Mehta et al., 2013), though more recently it was concluded that lampreys share the 2R duplications with jawed vertebrates after comparing the lamprey genome with a reconstructed ancestral *Amniota* genome (Sacerdot et al., 2018). Irrespective, it is clear lampreys do have more copies in many transcription factor gene families than amphioxus or sea squirts. Many of the jawed vertebrate paralogues are expressed by different cell populations in the spinal cord (Figure 3, Table2), as assessed by in situ hybridisation (summarised by (Alaynick et al., 2011; Lai et al., 2016)) and more recently by single cell sequencing (Delile et al., 2019). Some of this could represent subfunctionalisation of original broader expression patterns into subdomains, however some must also be neofunctionalisation and/or specialisation, for example the paralogues *Nkx6.1* and *Nkx6.2*, and *Dbx1* and *Dbx2,* overlap but are functionally distinct, setting up the interneuron progenitor zones p0, p1 and p2 (Alaynick et al., 2011). This would not be possible in amphioxus, with only one copy of each, and the duplication and resolution of *Nkx6* and *Dbx* into interacting pairs may have allowed the evolution of new interneuron domains.

9. Conclusions/Future perspectives: evolutionary change in spinal cord patterning

Comparing spinal cord development across the three chordate lineages (Figure 1) reveals both conservation and change. The general AP and DV polarisation of the spinal cord is conserved, with signalling molecules expressed by homologous tissues in similar positions (Figure 2). From this we can infer the common ancestor of vertebrates had tailbud mediated posterior growth, a DV axis framed by cells expressing Hh, Bmp and Wnt signals, and an AP

axis framed by RA and FGF. Downstream from these signals conservation is less apparent, and differences between lineages lead us to hypothesise a set of evolutionary innovations that underlie vertebrate spinal cord development.

First, considering the AP axis, RA has a role in regulating Hox in both amphioxus and *Ciona*, showing this was likely present in the chordate common ancestor. Wnt signalling has a more ancient role in posterior specification and expression of Wnt ligand genes in amphioxus is consistent with a role in Hox regulation (though this has yet to be experimentally demonstrated). FGF has a role in posterior regulation in both amphioxus and *Ciona*, showing this is also likely ancestral, though it is not yet clear how much of an influence it had on the Hox genes. In this context it may be notable that comparison of vertebrate and amphioxus Hox cluster regulation suggests an important difference in the regulatory regions that lie outside the cluster (Acemel et al., 2016). This suggests aspects of FGF regulation of Hox may be vertebrate-specific. Understanding whether and how FGF regulates Hox in lamprey and amphioxus would resolve this.

Second, patterning downstream of DV signals looks to be more elaborate in vertebrates. This suggests differences in the GRNs responsible for patterning and differentiation of neural cells. This might reflect new roles for divergent paralogues following 2R duplication, incorporation of new genes into GRNs, and/or new regulatory connections between genes already involved in spinal cord development in the common ancestor (Marletaz et al., 2018). Understanding the regulatory connections between signals and targets in lamprey and amphioxus, and between transcription factors in the DV GRN, would answer this.

Third, progenitor cell maintenance and ML organisation look quite different between vertebrates and other chordates. Lampreys are similar to jawed vertebrates, with ventricular progenitor cells divided into progenitor zones with proliferation under Notch regulation, and

giving rise to subset populations of neurons and glia patterned by DV signals. These were therefore present in some form in the common ancestor of vertebrates, however *Ciona* and amphioxus show no ventricular progenitor cell layer in the spinal cord, and hence no progenitor cell zones. This suggests that a major innovation in vertebrate spinal cord evolution was the origin of a cohesive ventricular progenitor cell pool, able to produce cells over a long period of development. Understanding this third change means comparing how progenitor cell maintenance and proliferation are controlled in vertebrates and other chordates; challenging at present since neither is well understood.

Furthermore, as discussed above in Section 5, proliferation and DV patterning are linked in vertebrates. A simple hypothesis arising form this is that, primitively, DV signals such as Hh directly regulated differentiation of early neural plate cells into specific cell types. In vertebrates this has evolved by insertion of a progenitor cell stage with new regulatory connections between patterning and proliferation. Evolution of the DV GRN would parallel this, incorporating new paralogues and new connections, and perhaps generating a system with the feedback and cross-repressive properties allowing the sudden state changes and scaling necessary to divide a spinal cord into discrete zones of cells.

While these hypotheses are speculative, they make predictions about ancestral regulatory connections and are hence testable by dissecting gene regulation in multiple lineages. With the recent development of methods for manipulating genes and regulatory elements in amphioxus and lamprey (Nikitina et al., 2009; Liu et al., 2013; Feng et al., 2014; Li et al., 2014a; Parker et al., 2014; Square et al., 2015; Zu et al., 2016), plus application of genome-wide methods to identify regulatory landscapes (Marletaz et al., 2018), this is rapidly becoming tractable.

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Figure 1. Chordate phylogeny and developing spinal cord anatomy.

Amphioxus is a member of the earliest diverging chordate lineage, and has a relatively simple spinal cord region that is just one cell thick. Tunicates, such as the sea squirt *Ciona*, have highly reduced spinal cord regions with just 4 cells in cross section and no neurons. Vertebrates have much more complex spinal cords, with more cells across the dorsal ventral axis, and multiple cell layers across the medial lateral axis. Lamprey and jawed vertebrate spinal cord diagrams are schematic, and differences are discussed more below. Diagrams adapted from (Lara-Ramirez et al., 2019).

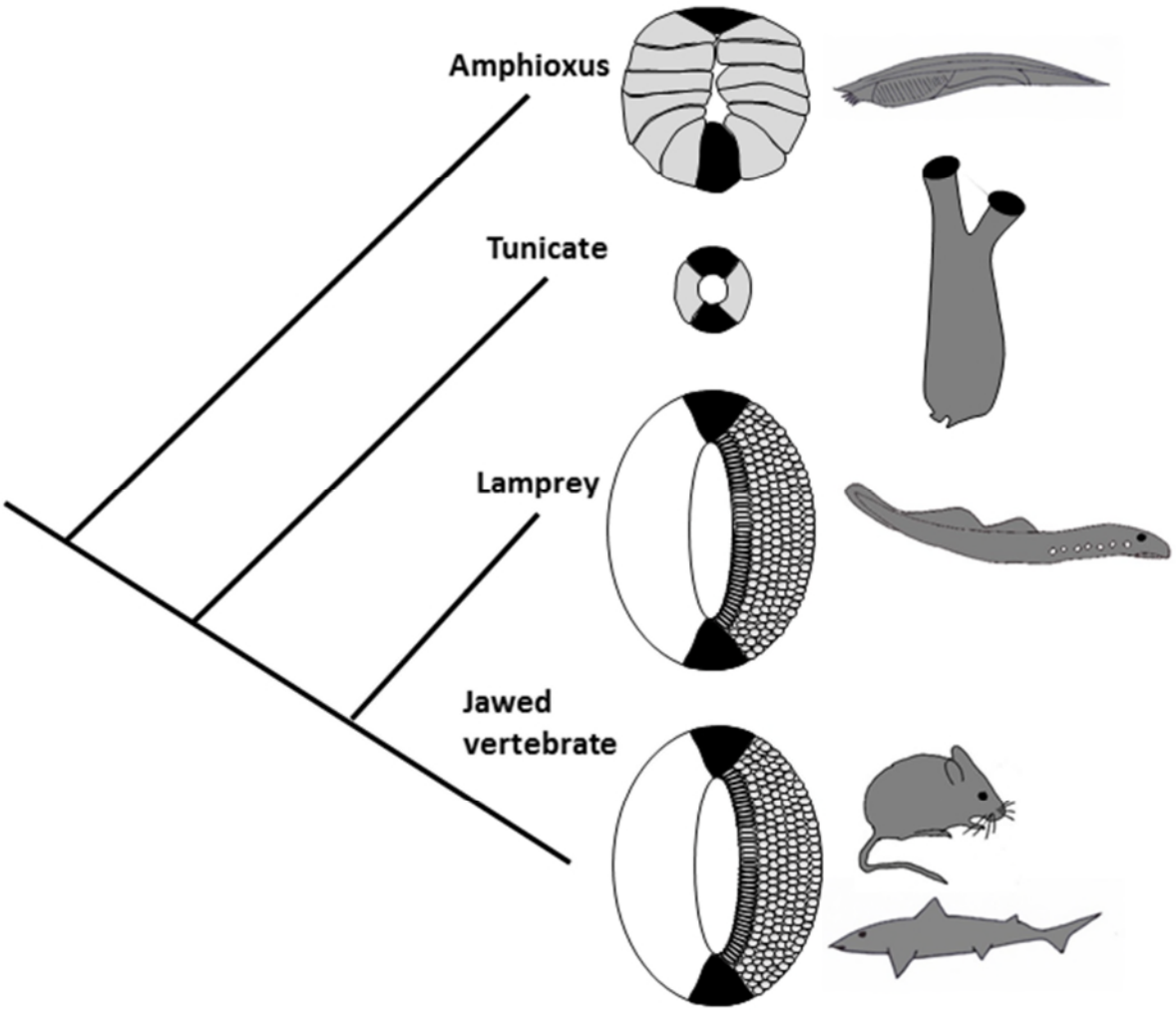

Figure 2. Signal sources in spinal cord patterning.

Schematic diagrams of gnathostome, lamprey and amphioxus spinal cords showing relevant expression of signalling molecules. Details and data sources as follows.

Hh in the floor plate in chordates. References: Gnathostomes; Mouse (Bai et al., 2002); Xenopus (Koide et al., 2006); Chick and zebrafish (Al Oustah et al., 2014); Lizard (Sun et al., 2018); Skate (Gillis and Hall, 2016). Lamprey; (Sugahara et al., 2011). Amphioxus; (Shimeld, 1999; Hu et al., 2017).

BMP in the roofplate in gnathostomes, dorsal neural tube and somites in lamprey, tailbud in amphioxus. References: Gnathostomes; Mouse, *BMP2*, *BMP6*, *BMP7*, *Gdf7* are expressed in roofplate (Augsburger et al., 1999; Butler and Dodd, 2003; Duval et al., 2014; Namm et al., 2015), *BMP4* in the whole spinal cord E9.5 then restricted to dorsal ectoderm by E10.5 (Hu et al., 2004; Andrews et al., 2017). Chick, *BMP4*, *BMP7* and *Gdf7* are in the roofplate (Chizhikov and Millen, 2004; Nitzan et al., 2016; Andrews et al., 2017), BMP6 expressed through out spinal cord except roofplate (Andrews et al., 2017). Lizard, BMP4 in the roofplate (Sun et al., 2018). Zebrafish, *BMP4* and *BMP6* in the dorsal epidermis (Dick et al., 1999; Thisse and Thisse, 2004; Merkes et al., 2015), *BMP7a* in the tailbud (Schmid et al., 2000), *BMP7b* in the pronephric duct, dorso-ventral tail fin (Shawi and Serluca, 2008). *Gdf7* in the head cartilage, dorsal aorta and pronephric duct (Davidson et al., 1999). Lamprey; *BMP2/4* in dorsal neural tube and somites (McCauley and Bronner‐Fraser, 2004). Amphioxus; *BMP2/4* in the tailbud (Panopoulou et al., 1998).

Canonical Wnt in the roofplate in gnathostomes and tailbud in amphioxus, no data on lamprey. References: Gnathostomes; Mouse, *Wnt1*, *Wnt3* and *Wnt3a* in the roofplate (Parr et al., 1993; Shinozuka et al., 2019); Chick, *Wnt1* and *Wnt3a* (Alvarez-Medina et al., 2008); Zebrafish, *Wnt1* and *Wnt3* in the roof plate (Tanaka et al., 2012; Duncan et al., 2015), *Wnt3a* in the tailbud (Fior et al., 2012). Lamprey; data only on non-canonical *Wnt5* which is expressed across the DV spinal cord (Guerin et al., 2009). Amphioxus; *Wnt1* in the tailbud, *Wnt3* in the tail fin and distributed along spinal cord (Somorjai et al., 2018).

Notch in the ventricular zone in vertebrates and in the tailbud in amphioxus. References: Gnathostomes; Mouse, (Machado et al., 2014); Chick, (Hämmerle and Tejedor, 2007);

Zebrafish, ventricular zone *notch* and *delta* (Appel et al., 2001; Kim et al., 2008). Lamprey; (Guerin et al., 2009; Lara-Ramirez et al., 2019). Amphioxus; (Holland et al., 2001).

RA from vertebrate somites and roofplate in gnathostomes and dorsal neurons in lamprey (Castillo et al., 2010), no data in amphioxus.

FGF in the tailbud of gnathostomes and amphioxus, no data on lamprey. References: Gnathostomes; Mouse, *Fgf3*, *Fgf4*, *Fgf8* and *Fgf17* in the tailbud (Wahl et al., 2007); Chick, *Fgf8* in the posterior neural plate and presomitic mesoderm (Bertrand et al., 2000); *Xenopus*, *Fgf8* and *Fgf20* in the tailbud (Lin and Slack, 2008); Zebrafish, *Fgf8* in the tailbud and posterior somites (Fior et al., 2012). Amphioxus; *Fgf9/16/20* patchily expressed in the neural tube, *FgfE* in a different patch of the neural tube, *FgfC* in the tailbud (Bertrand et al., 2011).

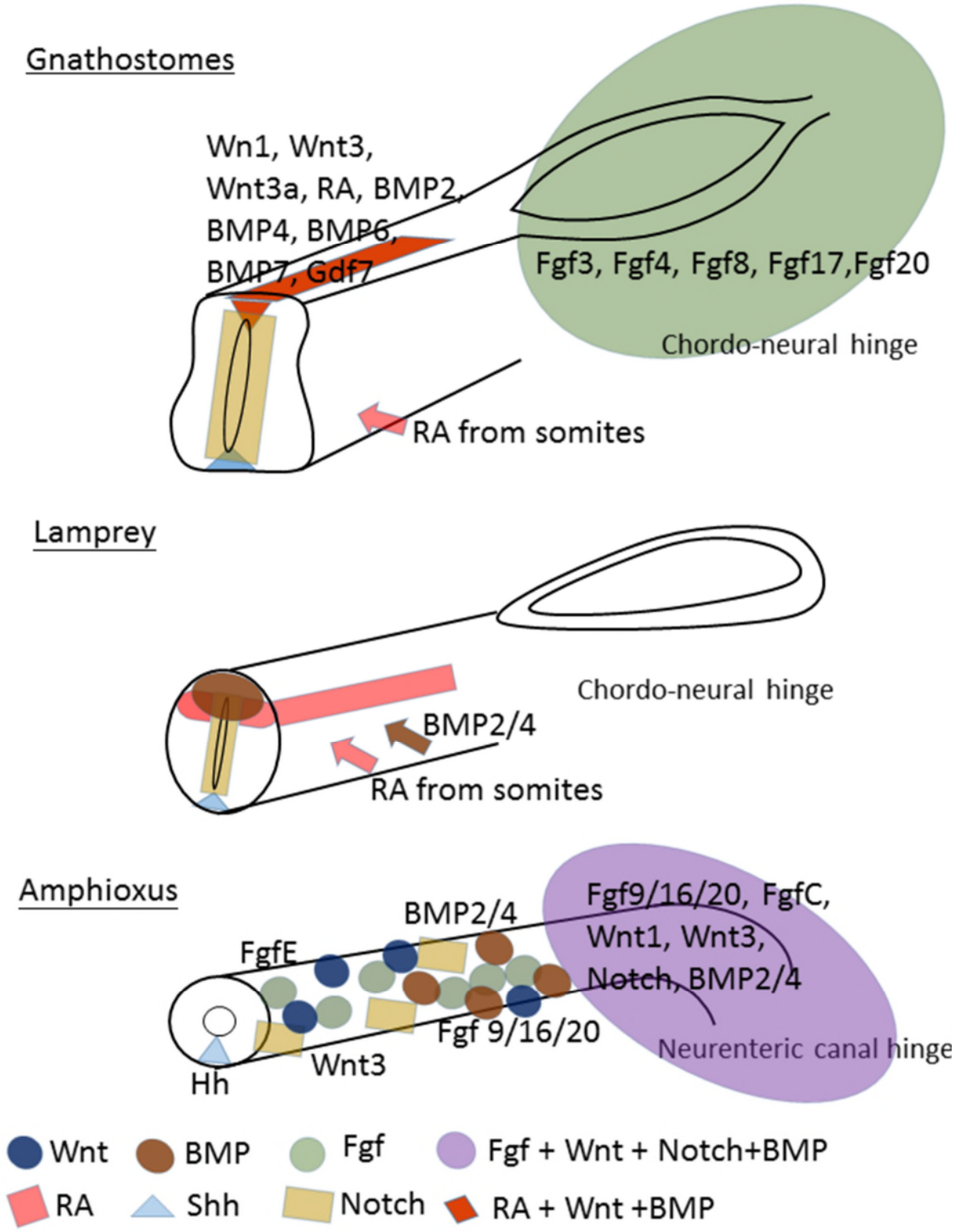

Figure 3. DV patterning of the vertebrate spinal cord.

To the right is a schematic cross section of a spinal cord, with signalling molecules and sources shown on one side, and the 11 progenitor zones shown on the other. To the left are the domains of expression of some of the transcription factor genes involved in DV patterning of the progenitor zones. Paralogue pairs are a similar shade of grey. Not all known genes are shown, and it should be noted that many of the domains of expression are dynamic, particularly in early development when pattern is being established, and that their strength of expression may vary within their domain of expression. For a more detailed recent exploration, see Lai et al (2016).

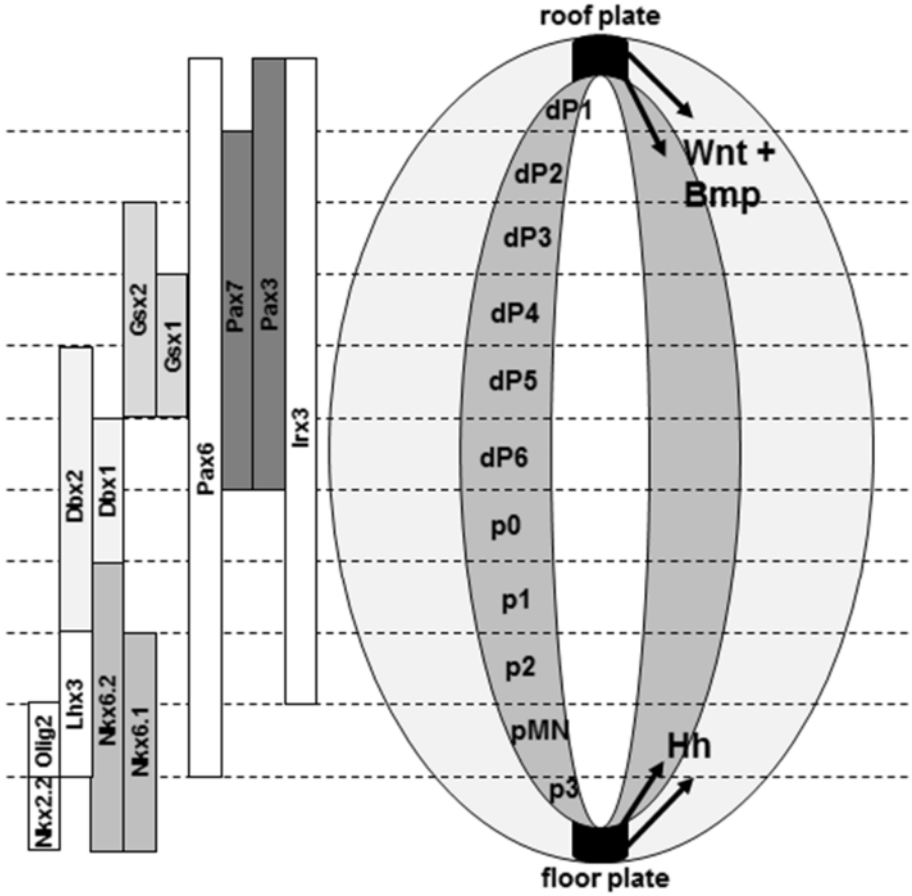

Figure 4. Cross sections of adult spinal cords showing anatomy and cell types

A simple phylogenetic tree on the left shows the evolutionary relationships of the taxa. Adult spinal cord cross-section anatomy and cell type are shown on the right. Each type of neuron

is only marked in one of either hemisegment. The dots only represent the general distribution and presence of the types of neurons, and not the numbers of expressing cells. Tunicates are not shown, as their spinal cord regresses before they reach adulthood. Data sources: Amphioxus**;** GABAergic neuron (Anadón et al., 1998), serotonergic neuron (Candiani et al., 2001), dopaminergic neurons not found in spinal cord (Moret et al., 2004), no data on other types of neurons. Lamprey**;** glutamatergic, glutamate-glycine, glutamate-GABA neurons (Fernández-López et al., 2012), dopamine-GABA neurons (Barreiro-Iglesias et al., 2009b), dopamine neurons (Fernández-López et al., 2015), dopamine-glutamate neurons (Fernández-López et al., 2017), aspartate, aspartate-GABA neurons (Villar-Cerviño et al., 2014), glycine, glycine-GABA neurons (Villar-Cerviño et al., 2008), cholinergic neurons (Quinlan and Buchanan, 2008). Larval data instead of adult for serotoninergic and GABAergic neurons (Barreiro-Iglesias et al., 2009a; Cornide-Petronio et al., 2014).

Zebrafish; (Pedroni and Ampatzis, 2019).

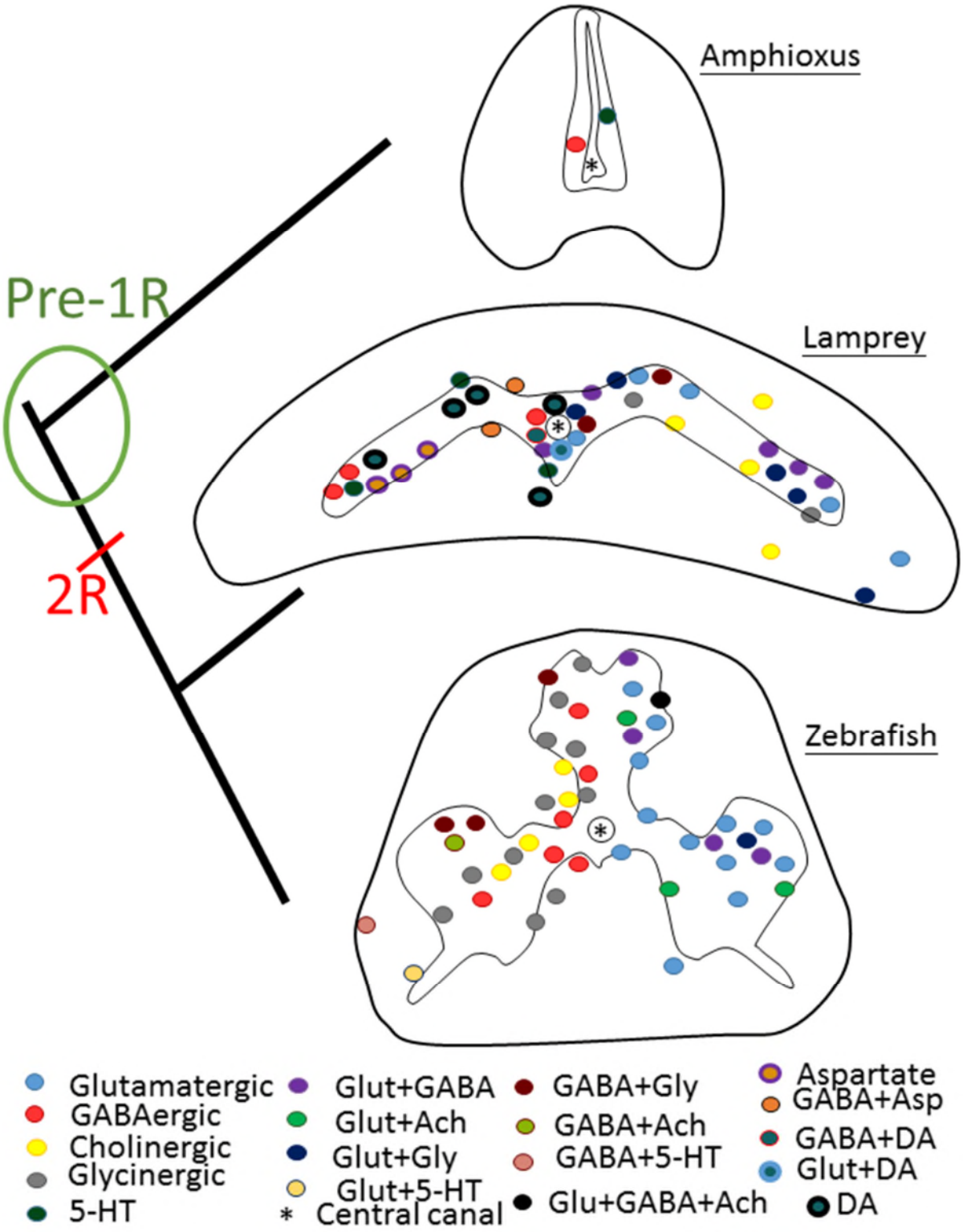

Table 1. Common Types of neurotransmitters

Excitatory	**Inhibitory**	**Modulatory**
Glutamate	Glycine	Acetylcholine

Aspartate	GABA	Noradrenaline
Nitric Oxide	Serotonin	
	Dopamine	

Table 2. Paralogues performing different roles in gnathostomes

Gene family	Types of paralogue divergence	Paralogue expression domain(s)	Reference
Neurog	Specialisation,	*Neurog2* dP2-pMN	(Timmer et al., 2002; Lee et al., 2003; Kriks et al., 2005)
	Neofunctionalsation	*Neurog1* dP2 *Neurog3* p3	
Msx	Specialisation	*Msx1* roofplate-dP5 *Msx2* roofplate-dP1	(Timmer et al., 2002)

Pax3/7	Specialisation	*Pax3* dP1-dP6 *Pax7* dP2-dP6	(Moore et al., 2013; Gard et al., 2017)
Pax2/5/8	Subfunctionalisation	*Pax2* dI4, dI6-V1 *Pax8* dI4, dI6-V1 (Batista and Lewis, 2008)	(Batista and Lewis, 2008)
Gata2/3	Specialisation	*Gata2* p2, V2 *Gata3* V2	*(Nardelli et al., 1999)*
Gsx	Specialisation	*Gsx2* dP3-dP5 *Gsx1* dP4-dP5	(Kriks et al., 2005)
Dbx	Specialisation	*Dbx2* dP5-p1 *Dbx1* dP6-p0	(Timmer et al., 2002)
Nkx6	Specialisation	*Nkx6.2* p1-floorplate *Nkx6.1* p2-floorplate	(Briscoe et al., 2000)
Olig	Neofunctionalisation	*Olig3* dP1-3, p0,p2,p3,V3	(Fu et al., 2002; Liu et al., 2014)

		Olig2 pMN	
Barhl	Redundancy	*Barhl1* dI1 *Barhl2* dI1	(Bulfone et al., 2000; Ding et al., 2012)
Islet1/2	Neofunctionalisation	*Islet1* dI3,MN *Islet2* MN	(Hutchinson and Eisen, 2006; Song et al., 2009)
Lhx1/5	Redundancy	*Lhx1* dI2, dI4, dI6-V1 *Lhx5* dI2, dI4, dI6-V1	(Pillai et al., 2007)
Lhx2/9	Redundancy	*Lhx2* dI1 *Lhx9* dI1	(Wilson et al., 2008)
Evx1/2	Neofunctionalisation	*Evx1* dI1, V0 *Evx2* V0	(Moran-Rivard et al., 2001; Juárez-Morales et al., 2016)
Tlx1/3	Neofunctionalisation	*Tlx3* dI3, dI5 *Tlx1* dI5	(Logan et al., 1998; Qian et al., 2002;

			Borromeo et al., 2014)