© 2019. Published by The Company of Biologists Ltd | Development (2019) 146, dev166595. doi:10.1242/dev.166595

RESEARCH ARTICLE

A Notch-regulated proliferative stem cell zone in the developing spinal cord is an ancestral vertebrate trait

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ABSTRACT

Vertebrates have evolved the most sophisticated nervous systems we know. These differ from the nervous systems of invertebrates in several ways, including the evolution of new cell types, and the emergence and elaboration of patterning mechanisms to organise cells in time and space. Vertebrates also generally have many more cells in their central nervous systems than invertebrates, and an increase in neural cell number may have contributed to the sophisticated anatomy of the brain and spinal cord. Here, we study how increased cell number evolved in the vertebrate central nervous system, investigating the regulation of cell proliferation in the lamprey spinal cord. Markers of proliferation show that a ventricular progenitor zone is found throughout the lamprey spinal cord. We show that inhibition of Notch signalling disrupts the maintenance of this zone. When Notch is blocked, progenitor cells differentiate precociously, the proliferative ventricular zone is lost and differentiation markers become expressed throughout the spinal cord. Comparison with other chordates suggests that the emergence of a persistent Notch-regulated proliferative progenitor zone was a crucial step for the evolution of vertebrate spinal cord complexity.

KEY WORDS: Lamprey, Notch signalling, Evolution, Proliferation, PCNA, Coe, Spinal cord

INTRODUCTION

The vertebrate spinal cord develops a precise pattern of neurons and glia under the control of multiple signalling pathways. Across the dorsal-ventral (DV) axis, ventral Hedgehog signalling and dorsal Bmp and Wnt signalling coordinate the formation of different neural populations (Gouti et al., 2015; Le Dréau and Martí, 2012). Differentiation along the anterior-posterior (AP) axis is regulated by a balance between anterior retinoic acid (RA) signalling and posterior FGF signalling (Diez del Corral et al., 2003). As the embryo elongates, the interface between these signals moves posteriorly, leading to a wave of cell differentiation along the spinal cord. Across the medial-lateral (ML) axis, cells close to the lumen remain in a proliferative progenitor state, forming the ventricular zone of the spinal cord. Other cells migrate laterally and differentiate (Gouti et al., 2015). Spinal cord cells thus integrate information across all three spatial axes for appropriate position-specific differentiation.

The Notch signalling pathway regulates numerous aspects of vertebrate development, and in the spinal cord it regulates neurogenesis by maintaining ventricular cells in a proliferative progenitor (stem cell) state (Myat et al., 1996). Notch signalling relies on cell-cell contact and is activated by binding of the transmembrane proteins Delta and Serrate/Jagged to Notch receptors on an adjacent cell. Upon ligand binding, Notch suffers two proteolytic cleavages; the first is catalysed by ADAM-family metalloproteases, while the second is carried out by the γ-secretase enzyme complex (Bray, 2006; Kopan and Ilagan, 2009). This releases the Notch intracellular domain (NICD), which translocates to the nucleus to promote transcription of target genes in combination with the transcription factor CSL (Bray, 2006; Fischer and Gessler, 2007). The best-known direct targets of NICD/CSL are the basic helix-loop-helix (bHLH) transcription factors of the Hes family, which exert an inhibitory role on neuronal differentiation by acting as antagonists to neural differentiation-promoting bHLH genes, including the neurogenin, atonal, ASCL and COE families. Experimental manipulation of Notch signalling in mice, *Xenopus* and zebrafish has shown that loss of Notch signalling causes cells to differentiate prematurely, depleting the progenitor pool (Appel et al., 2001; Wettstein et al., 1997). Thus, continued Notch signalling seems necessary to keep a progenitor pool over developmental time, and hence for spinal cords to develop their characteristic large number of cells. These progenitor cell populations may also be the source of adult neural stem cell populations (reviewed by Grandel and Brand, 2013).

A long-standing question in evolutionary biology is explaining how the complex central nervous system (CNS) of vertebrates evolved. Many studies have approached this by asking how neural patterning is regulated in vertebrates and in their nearest invertebrate relatives, cephalochordates (amphioxus) and tunicates (e.g. Albuixech-Crespo et al., 2017a,b; Holland et al., 2013). These studies have revealed patterning differences between vertebrates and these invertebrate lineages, explaining some of the complexity seen in the vertebrate nervous system. However, the complexity of patterning is only part of what makes vertebrates distinct, as vertebrates also have many more cells in their CNS than tunicates, cephalochordates and most other invertebrates.

Evolving more cells in an organ system could happen by several routes, although given that in vertebrate model species these cells develop from a ventricular progenitor pool, a simple hypothesis explaining the evolution of extra vertebrate neural cells is the evolution of mechanisms to generate and/or maintain these progenitors in significant numbers over an extended period of development. A prediction of this hypothesis is that a lasting progenitor pool and its conserved molecular regulation should be absent in invertebrate chordates with simple neural tubes, but

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Received 10 April 2018; Accepted 26 November 2018

Development (2019) 146, dev166595. doi:10.1242/dev.166595

present in all vertebrates, including the earliest diverging lineage – the agnathans (Shimeld and Donoghue, 2012). Lampreys and hagfishes, the only living agnathans, split from the lineage leading to jawed vertebrates before the evolution of hinged jaws and paired appendages, though their adult central nervous systems are large compared with those of cephalochordates and tunicates. Adult spinal cord anatomy in lampreys is relatively well studied, both as a model for the analysis of neural circuitry and for its capacity to regenerate following transection (Herman et al., 2018; Shifamn and Selzer, 2015). Much less is known about the embryonic and early larval development of the lamprey spinal cord. It is not known which signalling pathways govern cell patterning in this tissue and Notch signalling has not been experimentally tested, though some gene expression data indicate it might be involved, at least in the brain (Guérin et al., 2009).

To gain insight into the evolution of the vertebrate spinal cord and the involvement of Notch signalling in this event, we studied markers of neural cell proliferation and differentiation in lampreys, and whether these are regulated by Notch signalling. We show the entire lamprey spinal cord develops a ventricular progenitor zone that persists over an extended developmental period. We show maintenance of this progenitor zone is dependent on Notch signalling, and that compromised Notch signalling results in loss of the progenitor pool. Lost progenitors differentiate precociously. These data, when compared with data from other chordates, demonstrate that a CNS-wide proliferative ventricular progenitor zone evolved before the radiation of the jawed and jawless vertebrate lineages, but after their separation from extant invertebrate lineages. It is hence likely to be a vertebrate innovation. We also identify subtle differences in the outcome of Notch manipulation in the lamprey and jawed vertebrate lineages, suggesting additional evolutionary change following their divergence.

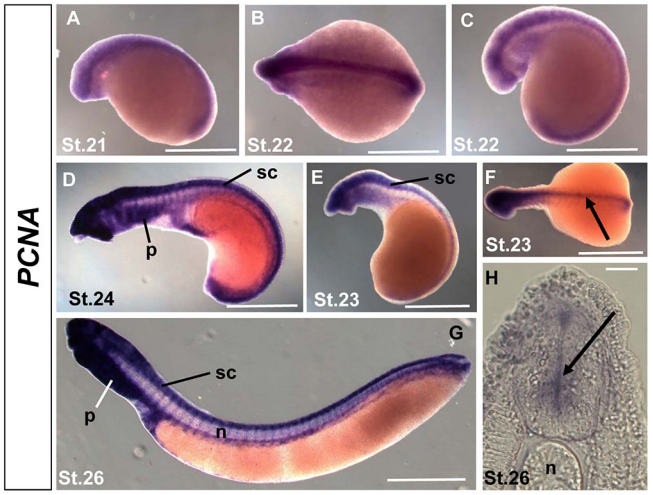

Fig. 1. *LpPCNA* expression identifies the lamprey spinal progenitor zone. (A-H) All embryos are in lateral view except B (dorsal view) and H (transverse section). Anterior is towards the left in all images except H. (A) At stage 21, *LpPCNA* expression is observed along the entire neural tube and in the pharyngeal region. (B,C) At stage 22, expression is maintained in the neural tube and extends into the developing pharyngeal arches. (D-F) At stages 23 and 24, expression increases in the entire neural tube and the pharyngeal region, and (F) can be seen to be restricted to the central (ventricular) region (arrow). (G) At stage 26, expression increases in the pharyngeal region, and expression is maintained in the entire neural tube. (H) A cross-section of a stage 26 embryo through the trunk region (dorsal towards the top) reveals *LpPCNA* expression in the ventricular zone (arrow). Apparent roof plate staining dorsal to the arrow is an imaging artefact and not stain. n, notochord; p, pharynx; sc, spinal cord. *LpMsi2* shows a similar pattern of expression (Fig. S5). Scale bars: 500 µm (A-G); 50 µm (H).

RESULTS

A ventricular proliferation zone is present throughout the lamprey spinal cord

To identify proliferating cells, we cloned the proliferation markers Proliferating Cell Nuclear Antigen (*PCNA*) and *Musashi* (*Msi*) from *Lampetra planeri* (*L. planeri* or *Lp*). *PCNA* is a co-factor of δ-polymerase, and is known to be expressed in the brain of a different lamprey species, *Lampetra fluviatilis* (Guérin et al., 2009). *Msi* genes encode RNA-binding proteins expressed in various stem cell populations, and one member of this family is also expressed in the *L. fluviatilis* brain (Guérin et al., 2009). Molecular phylogenetic analysis shows *LpPCNA* groups with other vertebrate *PCNA* sequences with strong support (Figs S1, S2). The *L. planeri Msi* sequence grouped within other chordate *Msi* sequences, and was most similar to jawed vertebrate *Msi2*; hence, we name this gene *LpMsi2* (Figs S3, S4).

We analysed the expression of *LpPCNA* and *LpMsi2* in normal embryos from Tahara (1988) stage 21 to stage 29 (Fig. 1, Fig. S5). At stage 21, *LpPCNA* is widely expressed in the protruding head and in the entire neural tube (Fig. 1A). Expression is maintained in the head and neural tube at stage 22 as the embryo grows, and at stages 23 and 24 expression is clearly observed in the pharyngeal region and becomes restricted to the ventricular region in the spinal cord (Fig. 1B-F). Strong expression persists through stages 25 and 26, with spinal cord expression restricted to the medial-most cells (Fig. 1G,H). This pattern essentially continues through stages 27-28, although brain and anterior spinal cord expression starts to weaken (data not shown). *LpMsi2* expression is similar to that of *PCNA* (Fig. S5). Thus, both proliferation marker genes are ventricularly expressed in the spinal cord over an extended developmental period, from stage 23 to at least stage 28, which spans about 2 weeks under normal developmental conditions. To confirm cells in the ventricular region were actively mitotic, we also stained sectioned embryos with an antibody to phophohistone 3 (pH3). This identified pH3-labelled cells in the ventricular region but not more laterally in the nervous system (Fig. S6). Fewer cells were detected than with *LpPCNA*; this was as expected considering that pH3 expression is restricted to the M phase of the cell cycle, whereas PCNA transcripts are present throughout the cell cycle.

Notch signalling is active and can be inhibited by DAPT treatment in lamprey embryos

Notch ligand expression has been provisionally described in two lamprey species, and, in the CNS, is broadly expressed early in development before becoming more confined to the ventricular region (Guérin et al., 2009; Kitt, 2013). Hairy/Hes genes lie immediately downstream of Notch signalling in many species (reviewed by Iso et al., 2003) and offer a route to understanding the strength of reception of Notch signalling. We cloned two *L. planeri* Hes homologs (*LpHesA* and *LpHesB*, Figs S7, S8). *LpHesB* expression was detected at stage 21 in the anterior CNS (Fig. 2A). Expression gradually increased and extended to most of the CNS at stages 22 and 23 (Fig. 2B-D). By stage 24, it had spread through the length of the CNS (Fig. 2E,F) and maintained this pattern until stage 28 (Fig. 2G-J and data not shown). Expression was also ventricularly restricted (Fig. 2F,G,J), as would be predicted from the location of proliferating cells.

To further investigate this, we used the Notch pathway inhibitor DAPT, which binds to and inactivates the γ-secretase complex, thus

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inhibiting Notch signalling by preventing release of NICD (Geling et al., 2002). This compound has been used as an inhibitor of Notch signalling in animals from chordates to cnidarians (e.g. Lu et al., 2012; Marlow et al., 2012) and can be added over defined developmental time windows. We treated embryos with DAPT between stages 24 (when brain patterning is well advanced but the spinal cord is still developing) and 26 (when the spinal cord has lengthened considerably). DAPT-treated embryos consistently bent backwards (e.g. Fig. 3A,B) upon DAPT treatment, while general morphology and anatomical relationships were otherwise maintained. If DAPT inhibits Notch signalling in lamprey embryos, it should manifest in a predictable change in Hes gene expression, specifically Hes gene expression should be lost from areas where Notch signalling is normally active. We hence assayed *LpHesB* gene expression in DAPT-treated and DMSO-treated control embryos. *LpHesB* expression appeared normal in control embryos (Fig. 3A,C,D). In DAPT-treated embryos, *LpHesB* expression in the brain is similar to controls, although shows some decrease in the midbrain and dorsal hindbrain (Fig. 3, compare C with F). However, expression was completely lost from the spinal cord (Fig. 3D,E,G,H). *LpHesB* expression was also lost from the tailbud (Fig. 3G,H). We conclude from these results that DAPT effectively blocks Notch signalling in the developing lamprey spinal cord under these conditions.

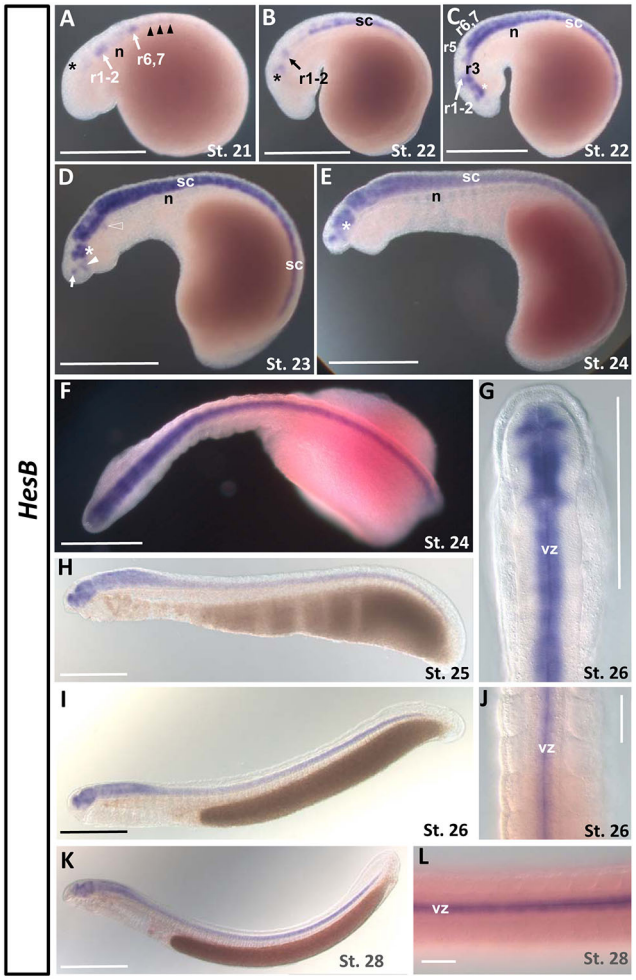

Fig. 2. Expression of *LpHesB* marks the progenitor zone in *L. planeri*. (A-E,H,I,K) Lateral views. (F,G,J,L) Dorsal views. Anterior is towards the left in all images except in G and J (anterior is towards the top). (G,J) Dorsal views of the head (G) and trunk (J) regions of a stage 26 embryo. (L) Dorsal view of a stage 28 embryo. (A) At stage 21, *LpHesB* is expressed in the forming neural tube. Strongest expression is seen in presumptive rhombomeres (r)1-r2 and r6-r7. Weaker expression is observed in presumptive midbrain (black asterisk) and spinal cord (black arrowheads). (B) At stage 22, expression increases in the midbrain (black asterisk) and considerably in the spinal cord. Expression is generally restricted to the ventral side of the neural tube. (C) In a slightly older stage 22 embryo, expression dramatically intensifies in the same expression domains, particularly in the midbrain (white asterisk) and spinal cord where expression extends posteriorly. Expression in r4 progressing dorsally starts to demarcate unstained r3 and r5. (D) At stage 23, expression has covered most of the rhombospinal region, except in the dorsal half of rhombomeres 3 and 5 (the open arrowhead points to r4). Expression is also present in a large territory in the ventral midbrain (white asterisk). Notably the MHB is not stained. At this stage, expression first appears in a discrete domain in the telencephalon (arrow) and ventral diencephalon (white arrowhead). (E,F) At stage 24, r3 and r5 are almost completely stained while the MHB remains unstained. Expression in the midbrain extends dorsally. At this stage, all expression along the neural tube is located in the ventricular zone (F). (H) At stage 25, expression covers the entire neural tube except the epiphysis and, as also seen at stage 26 (G,I,J), is restricted to the ventricular zone. (K,L) Stage 28 embryos in lateral and dorsal views, showing expression restricted to the ventricular zone. n, notochord; r, rhombomere; sc, spinal cord; vz, ventricular zone. Scale bars: 500 µm (A-I,K); 100 µm (J,L).

Notch signalling blockade leads to loss of spinal cord progenitors

We investigated whether Notch signalling also maintains the proliferative state of progenitors in the lamprey spinal cord by examining the expression of *LpPCNA* in control and DAPT-treated embryos. Upon DAPT treatment, *LpPCNA* was downregulated through the posterior hindbrain and anterior spinal cord (Fig. 4A,B, E,F). However, *LpPCNA* expression was maintained in the

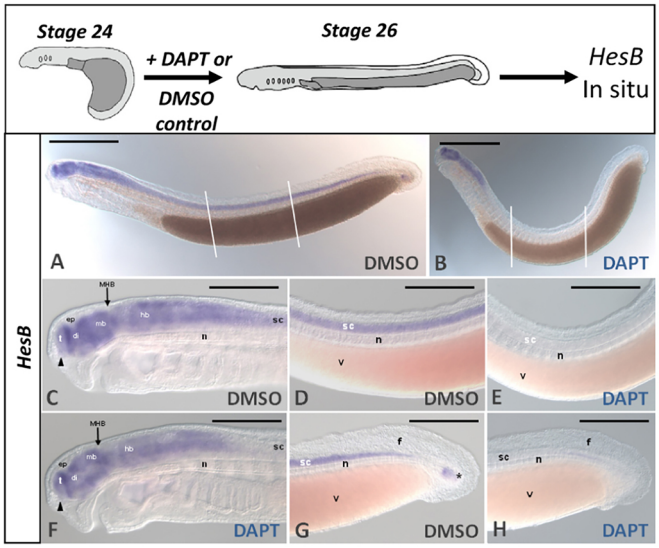

Fig. 3. Spinal cord *LpHesB* expression is lost when Notch signalling is inhibited. The diagrams at the top of the panel summarise the experimental design. (A,C,D,G) Control embryos. (B,E,F,H) DAPT-treated embryos. (D,E) Corresponding regions in control and DAPT-treated embryos, taken from between the lines in A,B, respectively. (G,H) Tail regions in control (G) and DAPT-treated (H) embryos. In all images anterior is towards the left. *LpHesB* is expressed in the entire CNS and in the tail bud (asterisk in G) in control embryos. Upon DAPT treatment, expression in the spinal cord is abolished (B,E) together with expression in the tail bud (H). Expression in the brain is maintained overall (arrowheads in C,F). ep, epiphysis; di, diencephalon; f, caudal fin; hb, hindbrain; mb, midbrain; MHB, midbrain-hindbrain boundary; n, notochord; sc, spinal cord; t, telencephalon; v, vitellum. Scale bars: 500 µm (A,B); 200 µm (C-H).

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posterior-most part of the spinal cord and tail bud region (Fig. 4A,B, G,H). In the brain, expression was reduced, although some expression was maintained in the forebrain, midbrain-hindbrain boundary (MHB) and posterior hindbrain, and expression was also strong in the pharynx (Fig. 4C,D).

Loss of *PCNA* expression suggests loss of proliferative progenitor cells; however, it could also reflect regulation of *PCNA* expression by Notch signalling without the cell progenitor to differentiated cell state being affected. To investigate this, we examined the number of pH3-labelled cells, finding they were significantly reduced in the anterior brain, hindbrain and spinal cord of DAPT-treated embryos when compared with controls (P=0.02, 0.008 and 0.001, respectively, t-test: Fig. S6). Moreover, nuclear staining with DAPI on sections revealed that DAPT treatment results in a loss of the orderly epithelial organization of the progenitor-containing ventricular zone, and a switch to the more loosely arranged and round cell morphology characteristic of differentiated cells (Fig. S6). Together, these data provide evidence for a loss of progenitor cells in the spinal cord of DAPT-treated embryos.

In jawed vertebrates, the progenitor zone is divided into DV zones, each formed by a specific population of proliferative cells and each marked by well-characterised combinations of transcription factor gene expression. Jawed vertebrate Olig genes mark two spinal cord regions: one ventral from which motor neurons will develop; and a dorsal region spanning three progenitor zones (Alaynick et al., 2011). Our searches of lamprey genome and transcriptome data identified two Olig genes, which we name *OligA* and *OligB* as they did not cluster robustly with jawed vertebrate Olig paralogues in molecular phylogenetic analysis (Figs S7, 8). We cloned lamprey *LpOligA* and examined its expression. In control embryos, *LpOligA* was expressed in three restricted domains of the brain (Fig. 5A): two patches were observed in the diencephalon, one just above the hypothalamus and the other slightly more dorsal, both adjacent to the zona limitans intrathalamica. The third domain of expression in the brain was in the dorsal hindbrain. In the spinal cord, two regions of expression were observed: a dorsal domain contiguous with that in the hindbrain and a ventral domain. This mirrors the combined expression of Olig paralogues in spinal cords of jawed vertebrates.

In DAPT-treated embryos, all three *LpOligA* expression domains in the brain were lost (Fig. 5D). Expression of both domains through the majority of the spinal cord was also lost, with the only remaining site a small population of cells in the very posterior region, near the tail bud (Fig. 5E,F). These data support the interpretation that loss of PCNA and pH3 in DAPT-treated embryos reflects a loss of the ventricular proliferative progenitor pool.

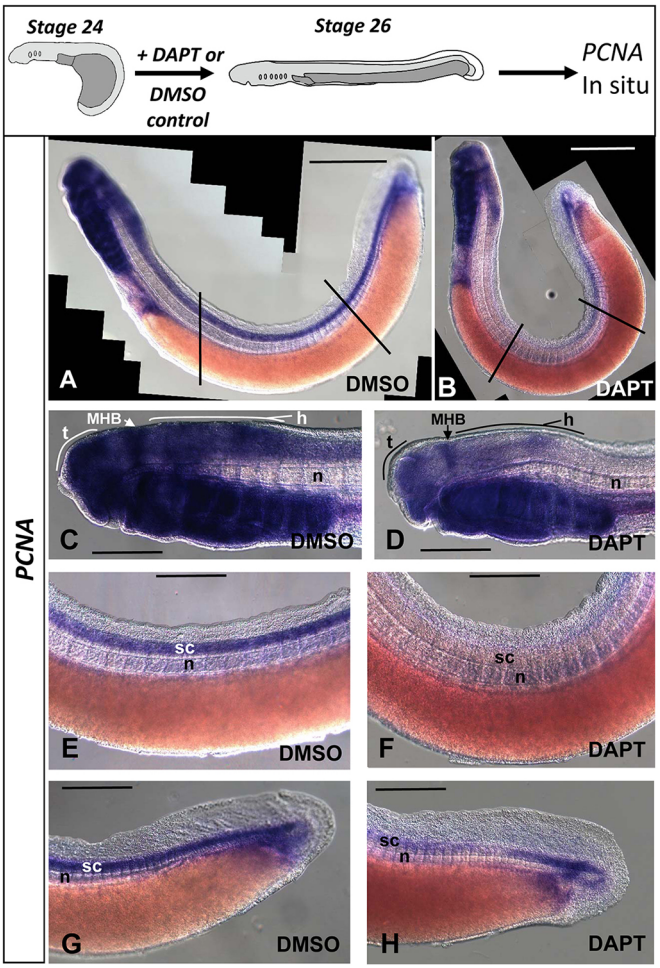

Fig. 4. Spinal cord expression of *LpPCNA* is lost when Notch signalling is inhibited. The diagrams at the top of the panel summarise the experimental design. Control embryos (A,C,E,G) are compared with DAPT-treated embryos (B,D,F,H). All photographs show embryos with the anterior towards the left. The regions within the lines in A and B show the parts of the spinal cord that have been imaged in E and F. Under DAPT, downregulation of PCNA is observed in the spinal cord, especially in the anterior part, and a decrease in expression in the brain is also evident. t, telencephalon; MHB, midbrain-hindbrain boundary; h, hindbrain; n, notochord; sc, spinal cord. A and B are collages of multiple smaller images of the same embryo. Scale bars: 500 µm (A,B); 200 µm (C-H).

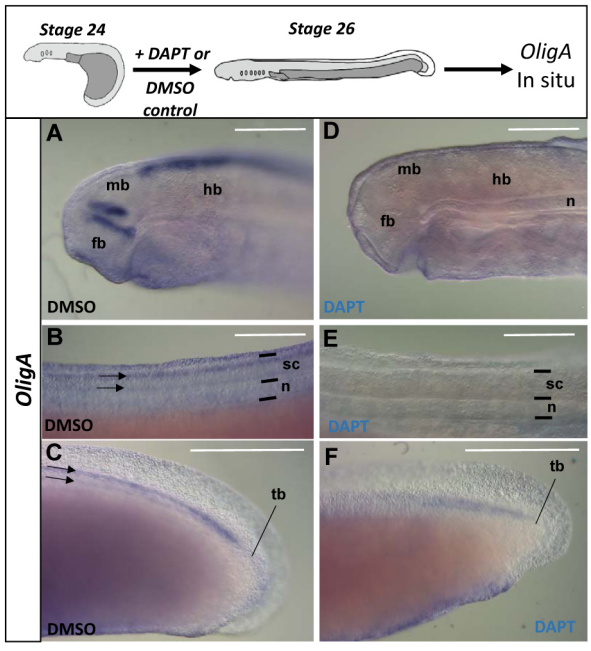

Fig. 5. Notch inhibition blocks *LpOligA* gene expression. The diagrams at the top of the panel summarise the experimental design. (A-F) Lateral views of the head (A,D), dorsal trunk (B,E) and tail (C,F). Anterior is towards the left in all images. (A-C) In control embryos, *LpOligA* expression is localised in two domains in the diencephalon, a dorsal stripe in the hindbrain and two stripes running along the length of the spinal cord (sc; arrows in B and C). These two stripes of expression appear to merge near the tailbud (tb). (D-F) In embryos treated with DAPT, all brain and spinal cord expression is lost, although expression persists in the tailbud. Black lines in B and E indicate the extent of the spinal cord and notochord (n). fb, forebrain; hb, hindbrain; mb, midbrain. Scale bars: 200 µm.

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Notch signalling blockade causes precocious differentiation in the lamprey spinal cord

To understand how the progenitor pool may have been lost, we examined the expression of the neuronal differentiation markers *LpCOE-A* and *LpCOE-B* (Figs 6 and 7). *LpCOE-A* and *LpCOE-B* are broadly expressed in differentiating neurons in both CNS and PNS of normal lamprey embryos (Lara-Ramírez et al., 2017), and in the spinal cord both genes are restricted to the more peripheral mantle layer. In DMSO-treated control embryos, both *LpCOE-A* (Fig. 6A,I,K) and *LpCOE-B* (Fig. 7A,I,K) were expressed in the peripheral region of the neural tube, as in normal development. In DAPT-treated embryos, no obvious changes of expression were observed in the head (Figs 6A-D and 7A-D). However, in the spinal cord, expression of both genes expanded in two ways: first expression expanded medially, fully occupying the ventricular spinal cord (Figs 6J,L and 7J,L); second, expression expanded to the posterior spinal cord into areas expressing little or no *LpCOE-A* or *LpCOE-B* in control embryos (Figs 6E-H and 7E-H). These data indicate that progenitor cells are differentiating precociously both in the ventricular zone and in posterior regions of the spinal cord following Notch blockade, leading to a loss of proliferative progenitors and an increase in cells expressing differentiation markers.

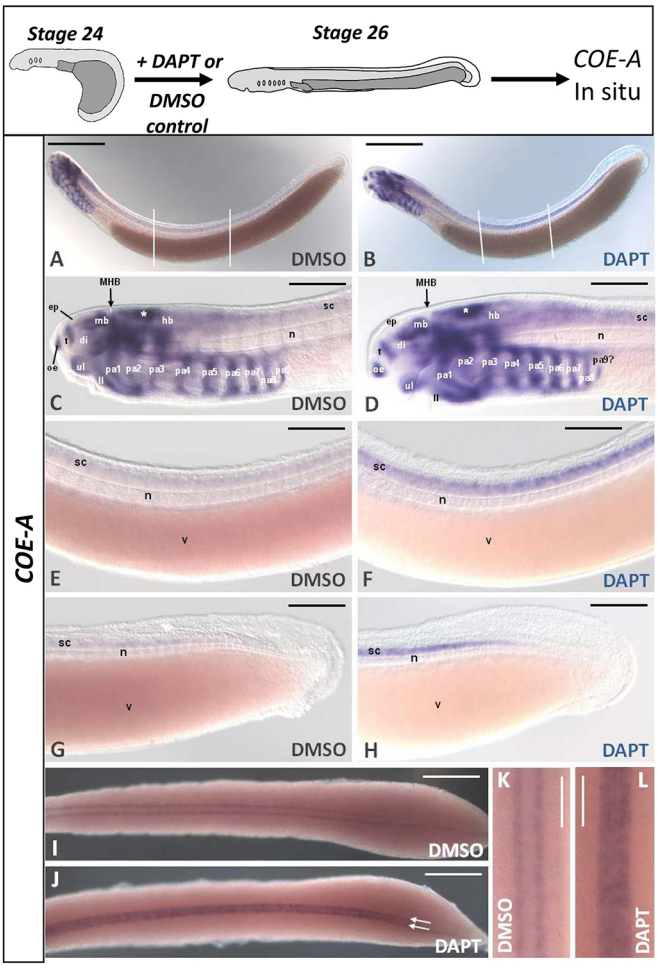

Fig. 6. *LpCOE-A* expression expands when Notch signalling is inhibited. The diagrams at the top of the panel summarise the experimental design. (A,C,E,G,I,K) Control embryos. (B,D,F,H,J,L) DAPT-treated embryos. (E,F) Photographs of corresponding regions in control and DAPT-treated embryos as indicated by the regions between the lines in A,B, respectively. (A-J) Anterior is towards the left; (K,L) anterior is towards the top. In the nervous system, *LpCOE-A* is expressed in the brain and faintly in the spinal cord in control embryos (A,C,E,G). Upon DAPT treatment, expression in the spinal cord is increased when compared with control embryos (B,D,F,H). (I-L) From a dorsal view, *LpCOE-A* is expressed in the spinal cord as two lateral stripes (I,K), whereas in DAPT-treated embryos it expands to the middle of the spinal cord (J,L). However, in the newly forming spinal cord at the posterior end, expression is seen as two lateral bands, as in control embryos (J, arrows). ep, epiphysis; di, diencephalon; hb, hindbrain; ll, lower lip; mb, midbrain; MHB, midbrain-hindbrain boundary; n, notochord; oe, olfactory epithelium; pa1-9, pharyngeal arch 1-9; sc, spinal cord; t, telencephalon; ul, upper lip; v, vitellum. Asterisks in C and D mark the dorsal hindbrain. Scale bars: 500 µm (A,B); 200 µm (C-H); 300 µm (I,J); 100 µm (K,L).

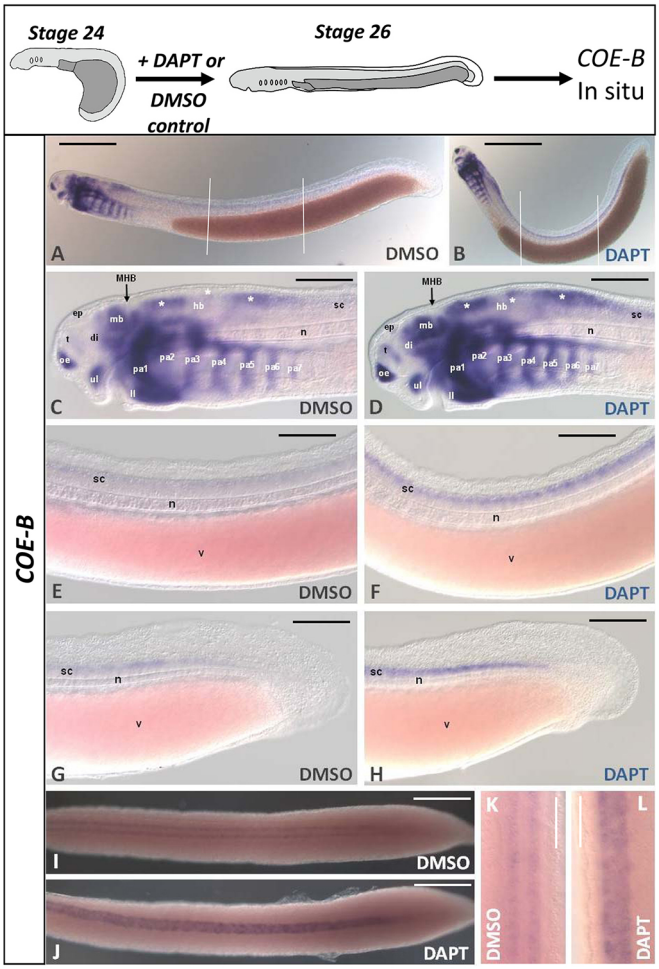

Fig. 7. *LpCOE-B* expression expands when Notch signalling is inhibited. The diagrams at the top of the panel summarise the experimental design. (A,C,E,G,I,K) Control embryos. (B,D,F,H,J,L) DAPT-treated embryos. (E,F) The corresponding regions in control and DAPT-treated embryos, indicated as the regions between the line in A,B, respectively. (A-H) Anterior is towards the left; (K,L) anterior is towards the top. In the nervous system, *LpCOE-B* expression is observed in restricted regions of the brain and cranial ganglia, and faintly in the spinal cord (A,C,E,G). Expression in the spinal cord is biased towards the dorsal side. Upon DAPT treatment, expression in the spinal cord is increased when compared with control embryos, although preserving its dorsal position (B,D,F,H). From a dorsal view, *LpCOE-B* in control embryos is seen in the spinal cord as two lateral stripes (I,K), whereas in DAPT-treated embryos expression expands into the middle of the spinal cord (J,L). ep, epiphysis; di, diencephalon; hb, hindbrain; ll, lower lip; mb, midbrain; MHB, midbrain-hindbrain boundary; n, notochord; oe, olfactory epithelium; pa1-7, pharyngeal arch 1-7; sc, spinal cord; t, telencephalon; ul, upper lip; v, vitellum. Asterisks in C and D mark the dorsal hindbrain. Scale bars: 500 µm (A,B); 200 µm (C-H); 300 µm (I,J); 100 µm (K,L).

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Blockade of Notch signalling alters neural patterning

The reduction of proliferative cells in the ventricular zone and their concomitant premature differentiation could indicate a simple 'speeding up' in the differentiation process while maintaining a normal distribution of cells. Alternatively, it could also result in an alteration of cell patterning. We noted that the different spatial distributions of *LpCOE-A* and *LpCOE-B* transcripts along the DV axis was generally maintained in DAPT-treated embryos, despite their ventricular expansion (Figs 6E,F and 7E,F). This preservation was most clear with *LpCOE-B*, which has a dorsally biased expression that was maintained upon DAPT treatment (Fig. 7E,F). However, increased expression of both COE genes in the anterior spinal cord also adopted a patchy pattern, suggesting the formation of clusters of differentiated cells (Figs 6F,J and 7F,J). We reasoned that this could indicate DAPT was interfering with lateral inhibition regulated by Notch, and to gain further insight into this we examined *LpNgnA*, which is expressed in the ventricular spinal cord in *L. planeri* (Lara-Ramírez et al., 2015). Expression of *LpNgnA* in DMSO-control embryos was as seen in wild-type embryos, i.e. localised in regions of the brain, the cranial ganglia and spinal cord (Lara-Ramírez et al., 2015) (Fig. 8A,C). In DAPT-treated embryos, expression appeared normal in the head (Fig. 8B,F); however, in the spinal cord, expression resolved into a series of discrete and widely spaced patches (Fig. 8B,E,H,I,K). These patches were absent in the anterior-most spinal cord but were progressively closer together towards the posterior end (Fig. 8E,H,I,K). These data suggest that, as well as differentiating precociously, local patterning of cells is also being affected.

To assess whether the cell differentiation induced by Notch blockade can proceed to normal neuronal maturation, we examined the axonal architecture in control and DAPT-treated embryos with an antibody to acetylated tubulin, a marker that has been previously shown to provide good definition of axonal tracts in lamprey embryos (e.g. Suzuki et al., 2015). Control embryos showed axonal architecture as previously described (Kuratani et al., 1997; Modrell et al., 2014; Murakami et al., 2004; Suzuki et al., 2016, 2015), with well-defined cranial nerves, prominent left and right longitudinal axonal tracts in the spinal cord, and segmentally organised nerves along the trunk (Fig. S9). In DAPT-treated embryos, overall neuronal architecture was similar to controls: the positioning of cranial nerves was the same, the longitudinal axonal tracts in the spinal cord were present and the segmental nerves in the trunk were similarly organised (Fig. S9).

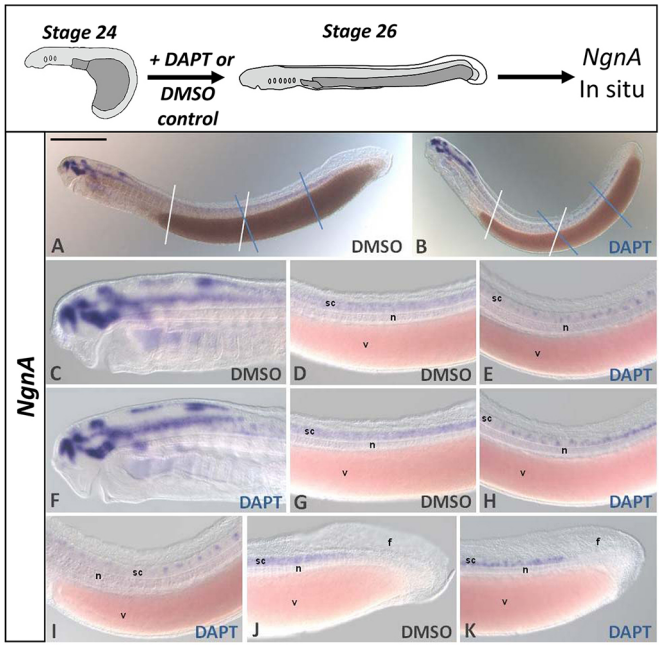

Fig. 8. Expression of *LpNgnA* in DAPT-treated embryos. The diagrams at the top of the panel summarise the experimental design. (A,C,D,G,J) Control embryos. (B,E,F,H,I,K) DAPT-treated embryos. (D,E) Photographs of corresponding regions in control and DAPT-treated embryos, indicated by the region between the white lines in A,B, respectively. (G,H) Photographs of corresponding regions in control and DAPT-treated embryos, indicated by the region between the blue lines in A,B, respectively. (I) A different embryo; the region of the trunk shown in this photograph overlaps with, but is anterior to, the region between the white lines in A,B. In all images, anterior is towards the left. *LpNgnA* is expressed in restricted regions of the brain in control embryos, as in normal embryos (A,C; Lara-Ramirez et al., 2015). In the spinal cord, *LpNgnA* is expressed relatively homogenously. Under DAPT treatment, spinal cord *LpNgnA* expression changes to clusters of cells all along the spinal cord (B,E,H, I). These clusters present an irregular organisation, being bigger towards the anterior and smaller towards the posterior. Additionally, they are more densely packed towards the posterior and terminate immediately before the end of the spinal cord (K). f, caudal fin; n, notochord; sc, spinal cord; v, vitellum. Scale bar (in A, applies to all panels): 500 µm (A,B); 200 µm (C-K).

DISCUSSION

The control of spatial and temporal patterning of the spinal cord of vertebrate model systems is relatively well understood, with RA, FGF, Bmp, Wnt and Hh signals providing AP and DV axial information, and Notch signalling participating in the development of the spinal cord by maintaining a ventricular proliferative zone of neural precursors (reviewed by Briscoe and Novitch, 2008). Less well-known is when and how such patterning and its resultant complexities evolved. In this study, we show that the Notch signalling is active in the spinal cord of a basally diverging vertebrate, where it regulates proliferation and differentiation. This identifies the Notch-dependent proliferative progenitor zone as a vertebrate characteristic, and we propose this is an important evolutionary difference to other chordates.

The lamprey spinal cord has a proliferative ventricular zone regulated by Notch signalling

Previous studies with antibodies and RNA probes have suggested a layer of proliferating cells may lie next to the lumen of the lamprey brain in embryos and larvae (Guérin et al., 2009; Villar-Cheda et al., 2006). It has also been reported that some proliferative cells can be identified in the ventricular spinal cord of larvae that are 2-5 years old (Vidal Pizarro et al., 2004), with activation of cell division also detected in 4-5 year old larvae after spinal cord injury (Zhang et al., 2014), although embryos and earlier larvae were not examined in either case. How proliferation is regulated has also not been determined. Our analysis of the expression of *LpPCNA*, pH3, *LpMsi2* and *LpHesB* corroborate the localisation of proliferating cells in the brain, and in addition portray a ventricular zone of proliferating cells in the embryonic spinal cord. This shows that lampreys maintain a ventricular progenitor zone of proliferative, undifferentiated cells throughout the developing CNS. This lasts for an extensive period of spinal cord development, more than 2 weeks under normal developmental conditions (Tahara, 1988). In addition, expression of the neural differentiation markers *LpCOE-A* and *LpCOE-B* marks a complementary mantle layer of differentiating cells along the entire neural tube. These data show the lamprey spinal cord resembles that of jawed vertebrates with respect to the relative placement of proliferative and differentiated cells. In particular, over a relatively long developmental time, lampreys

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maintain a proliferative stem cell zone filling the ventricular region of the spinal cord from post-neurulation stages until at least approaching the point the animal becomes a fully formed, free-living and feeding organism. This is a fundamental difference from neural development in invertebrate chordates, discussed in more detail below.

Given the presence of a ventricular progenitor cell population in the lamprey spinal cord, previously reported *Notch* gene expression (Guérin et al., 2009; Kitt, 2013) and the distribution of *LpHesB* expression (Fig. 2), we reasoned the lamprey progenitor zone may be Notch regulated. To test this, we turned to DAPT, as very early neural expression of *Notch* (Sauka-Spengler et al., 2007) precludes morpholino or simple gene editing-based approaches for examining its late developmental roles. Although we cannot exclude the possibility that DAPT has other effects on development than those mediated by Notch signalling, DAPT has been widely used as an inhibitor of Notch across many animal phyla (e.g. Lu et al., 2012; Marlow et al., 2012), its target presenillin is highly conserved in lampreys (Fig. S10) and the downregulation of *LpHesB* throughout the spinal cord of DAPT-treated lamprey embryos shows Notch signalling is affected.

Blocking Notch signalling in developing lamprey embryos results in the loss of proliferative ventricular cells, as visualised by loss of *LpHes*, *LpPCNA* and *LpOligA* expression, with complementary upregulation of *LpCOE-A* and *LpCOE-B* expanding into the ventricular zone. This indicates premature differentiation of these cells. The loss of both dorsal and ventral spinal expression domains of a marker of specific subsets of progenitor cells, *LpOligA*, supports this interpretation. Thus, we conclude that the balancing of a ventricular proliferative zone against a peripheral differentiating zone, mediated by Notch signalling, is conserved between lampreys and jawed vertebrates, and hence a character of the vertebrate common ancestor.

The evolutionary history of Olig genes has been studied previously (Li and Richardson, 2016), revealing *Olig2* and *Olig3* are paralogues dating from early vertebrate genome duplications, whereas *Olig1* is a tandem duplication deriving from *Olig2* and probably more recent (although still pre-dating the radiation of living jawed vertebrates). In jawed vertebrates, the dorsal and ventral Olig-positive progenitor zones are marked by *Olig3* and *Olig2*, respectively. These domains seem to be encompassed by *LpOligA*, suggesting subfunctionalisation in jawed vertebrates. Jawed vertebrate Olig gene expression also marks at least some differentiated cells that develop from the ventral progenitor domain, most prominently the myelinating oligodendrocytes from which the gene family takes its name. In particular, the role of *Olig1* is closely tied to oligodendrocyte differentiation (Li and Richardson, 2016). Although myelination itself is not found in jawless fish, the ventral progenitor domain in lampreys has been shown to produce glia, and gene expression and CRISPR/Cas9-mediated knockout indicate some conservation of regulation in this region (Yuan et al., 2018). We did not find *LpOligA* expression in this glial cell population or other mantle layer cells, explaining the absence of *LpOligA* expression in DAPT-treated embryos. However, we note that lampreys have a second Olig gene, *OligB* (Figs S7, S8). Our molecular phylogenetic analysis did not place lamprey *OligA* or *OligB* as orthologous to any specific jawed vertebrate Olig gene and they are not linked like *Olig1* and *Olig2*, so we cannot draw simple comparisons between genes. However, a possibility is that in lampreys Olig family subfunctionalisation differs to that in jawed vertebrates, with *OligB* having a role in ventral glial differentiation.

Not all aspects of spinal cord cell proliferation appear to be Notch regulated. First, we note that, the tail bud-associated domain of *LpHesB* expression is lost on DAPT treatment (Fig. 3G,H), while posterior expression of *LpOligA* (Fig. 5C,F) and *LpPCNA* (Fig. 4G,H) are maintained, and ectopic *LpCOE* expression does not extend into this region (Figs 6H and 7H). This shows these cells are Notch independent, and one possibility is that their proliferation and differentiation state are regulated by tail bud-derived signals such as FGF8, as reported for some jawed vertebrate model species (reviewed by Diez del Corral et al., 2003). Second, *LpNgnA* does not behave as a canonical proliferative zone gene. Its expression is not fully lost when Notch is blocked. Neither, as would be predicted from comparison with jawed vertebrates, is there a general, relatively homogenous, upregulation of expression (Geling et al., 2002; Nornes et al., 2008; Yang et al., 2006). Instead, when Notch is blocked in lamprey embryos, broader expression is lost but small clusters of *LpNgnA*-expressing cells emerge. This pattern is reminiscent of the effects of blocking Notch signalling in Notch-dependent lateral inhibition systems, and of the Ngn-dependent regulation of specific neuronal types in the vertebrate spinal cord (Korzh and Strähle, 2002; Nornes et al., 2008). The distribution of Ngn-expressing cells also bears a resemblance to what is observed in normal amphioxus development, something discussed in more detail below. Tubulin staining of control and DAPT-treated embryos did not reveal differences in axonal architecture, and tubulin staining did not expand into the ventricular zone in DAPT-treated embryos. This suggests that, at least for cells and structures revealed by this method, there are no major changes in axonal pathfinding after DAPT treatment and longitudinal axon tracts remained in the mantle region. However, phenotyping with further cell-type marker genes and comparison of neurotransmitter distribution between control and DAPT-treated embryos will be needed to understand what the *LpNgnA*-expressing cell clusters are, and whether other subtle differences in cell organisation have occurred.

Notch signalling, patterning and cell proliferation in the lamprey head

When comparing the effects of Notch inhibition in anterior regions of the neural tube, we noticed a difference to the spinal cord. Blocking Notch signalling resulted in complete loss of *LpHesB* expression from the spinal cord, and from the tailbud, but not from the brain. In particular, *LpHesB* expression in the forebrain, midbrain and anterior hindbrain did not appear much different between DAPT and control embryos, with only some minor changes in the midbrain and hindbrain observed. However, there was a clear transition in the response to DAPT visible around the hindbrain-spinal cord junction at about the level of the 5th pharyngeal slit (Fig. 3F). Despite this, *LpPCNA* expression was reduced in the brain (particularly in the midbrain and dorsal hindbrain) when Notch was blocked, $pH3^+$ cells were significantly reduced in number and *LpOligA* expression was completely lost. We note that the domains from which *LpOligA* is lost, in the midbrain and dorsal hindbrain, also match the areas where *LpPCNA* expression is most reduced, and where *LpHesB* expression appears affected. The complete loss of *LpOligA* shows differences between spinal cord and brain are unlikely to be explained by poor penetrance of the DAPT into the brain, and the significant reduction in the number of $pH3^+$ cells confirms it is having an impact of cell proliferation. The time window of DAPT treatment might be relevant, e.g. with respect to how actively cells in each are dividing or differentiating. A similar distinction of brain versus spinal cord sensitivity to Notch signalling has been suggested in the mouse based on embryos double-null mutant for *Presenilin-1* and *Presenilin-2*, in which *Shh* and *Nkx2.2* expression in the ventral neural tube is absent from the trunk but maintained in the head (Donoviel et al., 1999). These authors

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suggested that the effects of Presenilin loss are restricted to the trunk region. This raises the possibility of a conserved difference between the brain and the spinal cord in the response to Notch signalling, with the possibility that additional inputs maintain Hes expression and/or proliferation in parts of the brain. Further dissection of proliferating cell localisation, proliferation rates and differentiation in the lamprey head will be needed to resolve this.

The evolutionary origin of complexity in the vertebrate spinal cord: a hypothesis

All animals except sponges make a nervous system. Although there is anatomical variation in neurogenesis between animal lineages, there are also consistent aspects, probably controlled by conserved genetic modules that predate the radiation of bilaterians and may also be conserved with earlier-diverging lineages like the Cnidaria. For a detailed exploration of early nervous system development and evolution, see the comprehensive review by Hartenstein and Stollewerk (Hartenstein and Stollewerk, 2015). Briefly, embryos generally move through phases where broad competence to form neural progenitors is first defined, then neural progenitors are patterned and specified, followed by scope for progenitors to proliferate before differentiation. In some lineages progenitors may delaminate, migrate, ingress or, as in the chordate neural tube, invaginate. One conserved aspect is that cells receiving high levels of Notch signalling maintain high levels of Hes expression and remain undifferentiated, whereas cells receiving low levels of Notch progress towards differentiation.

The cephalochordates and tunicates are the closest living relatives to vertebrates, and the only invertebrates with neural tubes clearly homologous to those of vertebrates. Their neural tubes, however, are simpler and contain fewer cells than those of vertebrates. The ascidian larval central nervous system is composed of the sensory vesicle, the neck, the visceral or motor ganglion and the tail nerve cord (Lemaire et al., 2002; Meinertzhagen et al., 2004). Based on morphology and gene expression, the tail nerve cord is considered to be the equivalent of the vertebrate spinal cord (Wada and Satoh, 2001). However, the ascidian tail nerve cord is composed only of ciliated ependymal cells, distributed in a row of ventral keel cells, left and right lateral rows, and a dorsal row of capstone cells, thus four cells in cross-section (Fig. 9) (Lemaire et al., 2002; Meinertzhagen et al., 2004; Wada and Satoh, 2001). There is no active cell division in the posterior CNS beyond early development.

The amphioxus larval central nervous system is a tubular nerve cord and contains more cells than in tunicates, including neurons along its length. It presents a transient anterior swelling called the cerebral vesicle (hereafter called the brain) (Wicht and Lacalli, 2005). In larvae, the CNS posterior to the brain is made of a single layer of cells, surrounded by axon tracts (Bone, 1959; Lacalli and Kelly, 2002). Glia are present at or adjacent to the ventral midline (Lacalli and Kelly, 2002). Amphioxus spend a considerable period of time as a larva before metamorphosis, a process that involves reshaping of the head and pharynx (amongst other changes) but has not been studied in much detail at the molecular or cellular levels. Adult anatomy has been well documented, and a comprehensive review of nervous system anatomy has been produced by Niuewenhuys (Nieuwenhuys, 1998). In particular, Bone's 1960 study gave detailed insight into the distribution and type of cells through the CNS, identifying the positioning of various axon bundles and the location of cell bodies (Bone, 1960). The adult nervous system appears to be more complex than in larvae, with, for example, ventral somatic motor bundles, multiple different types of cells associated with the dorsal root and commissural cells, including

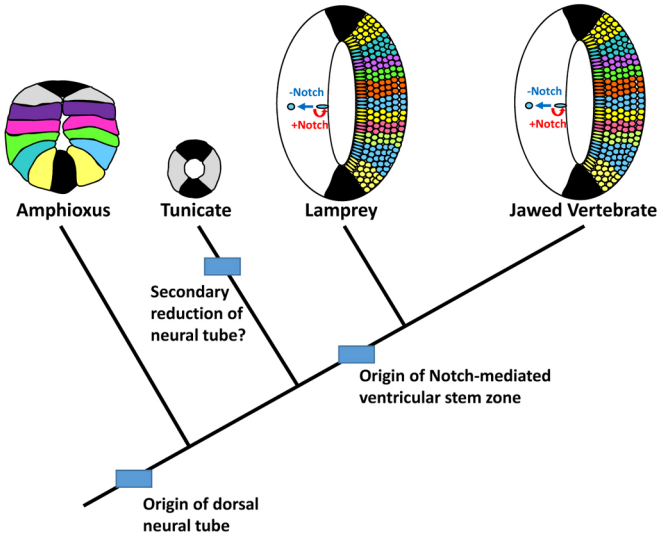

Fig. 9. A model for spinal progenitor evolution in chordates. The diagrams at the top show schematics of the spinal cord or equivalent regions of the four major chordate lineages. The floor plate and roof plate, considered homologous between all chordates and the sources of DV patterning signals (Corbo et al., 1997; Panopoulou et al., 1998; Shimeld, 1997, 1999), are shown in black. In amphioxus, scattered neurons of different types, some of which express marker genes found in specific subpopulations of vertebrate spinal cord cells, are found in the nerve cord posterior to the anterior swelling called the cerebral vesicle. In larvae, the neural tube appears to be one cell thick (Lacalli and Kelly, 2002), although may develop more cells in the anterior by the time adulthood is reached. Tunicates such as *Ciona* have only four rows of cells in the posterior neural tube and no neurons, although this is thought to be a secondary loss of complexity. Jawed vertebrates show two key differences: (1) cells are organised into DV zones, with all cells in a zone defined by the same transcription factor gene code; and (2) a Notch-regulated stem zone is present adjacent to the lumen of the neural tube. Our data show both are also present in lampreys, and hence we conclude they are a vertebrate innovation that underlies the increase in nerve cell number and consequent neural complexity seen in vertebrates. The depiction of identical lamprey and jawed vertebrate progenitor zone organisation is schematic. Although this study and others (Guérin et al., 2009; Kusakabe et al., 2011; Yuan et al., 2018) have shown similarities in patterning between the two lineages, we do not yet know if there are also important differences.

neurons that send processes through the neural tube lumen to make contralateral connections. However, cell bodies all appear to be ventricular, with no demarcation between cellular ventricular and mantle regions, as in vertebrates. The exception may be the brain, where more cells are found and some subdivision of cell types has been described (Nieuwenhuys, 1998), although their proliferation is unstudied. In summary, as well as the overall low cell number in the CNSs of both amphioxus and tunicates, a ventricular zone of neural progenitors and a complementary marginal zone of differentiated cells have not been described in either lineage, with the possible exception of the amphioxus brain where more study is needed.

Expression of Notch signalling pathway components and neural HLH genes has been analysed in ascidian tunicates and cephalochordates. In the ascidian *Ciona robusta* (formerly known as *Ciona intestinalis* type A), *Delta*, Hes, Ngn and COE genes are expressed in a small number of neural cells, mostly peripheral neural and sensory vesicle cells, with little or no expression in the tail nerve cord, and Notch expression persists into the nerve cord until the mid-tailbud stage (Imai et al., 2004; Mazet et al., 2005; Yamada et al., 2009). In the ascidian *Halocynthia roretzi*, Notch signalling appears to be lost from the tail nerve cord by the mid tail bud stage (Hori et al., 1997). In amphioxus, the expression of *Notch*, *Delta*,

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Hes, Ngn and COE genes has been found in the neural tube, including the region equivalent to the vertebrate spinal cord. *Notch* is strongly expressed in the neural tube at early neurula stages but is subsequently down regulated (Holland et al., 2001), and *Delta* expression is lost early in development from the region equivalent to the vertebrate spinal cord (Rasmussen et al., 2007). Ngn, Hes and COE genes mark scattered cells in the neural tube (Beaster-Jones et al., 2008; Holland et al., 2000; Mazet et al., 2004; Minguillon et al., 2003). Relevant gene expression data in amphioxus late larvae, metamorphs and adults has not been generated, although one report has indicated that Notch pathway components may be expressed during regeneration of the posterior body following amputation, a process that includes the formation of new tail CNS tissue (Somorjai, 2017).

Posterior CNS development in ascidians and amphioxus therefore differs from all vertebrates (including lampreys) in three ways: (1) neither has a ventricular proliferative progenitor pool; (2) in both lineages Notch signalling is maintained only through early development; and (3) the expression of neuronal HLH genes is confined to scattered individual cells. To this we can add the observation that many of the genes that define vertebrate DV progenitor zones, and the pools of neurons that develop from them, are also only expressed in scattered individual cells in amphioxus, including members of the Olig, *Prdm12*, *Evx*, *Engrailed* and Isl/Lhx gene families (Albuixech-Crespo et al., 2017b; Beaster-Jones et al., 2008; Ferrier et al., 2001; Holland et al., 1997; Jackman and Kimmel, 2002; Thelie et al., 2015). Furthermore, when Notch signalling is blocked in lamprey embryos, aspects of the resultant pattern of differentiating cells resemble what is observed in amphioxus, with patchy cells rather than clearly defined DV zones.

The vertebrate spinal cord is a far more complex structure than the equivalent in other chordates. Its development involves the production of a large number of different cell types, via a ventricular proliferative zone generating a peripheral zone of differentiated neurons over an extended period of development. Our data show this is present in lampreys, and hence a synapomorphic character of all living vertebrates. Thus, alongside elaboration of patterning mechanisms in early vertebrate evolution, we propose that the emergence of a Notch-regulated ventricular progenitor zone along the length of the CNS was a key step in vertebrate nervous system evolution (Fig. 9). In this model, a simple basal chordate nervous system was patterned across the DV axis directly into discrete cell types, marked by the expression of conserved transcription factor genes, such as Olig, *Prdm12*, *Evx* and Lhx. Notch signalling had only a transient early role, proliferation was curtailed and progenitor pools did not form. In vertebrates two connected innovations evolve; a ventricular progenitor zone creates more cells over a longer developmental time, and division of the progenitor region into DV pools forms a diversity of stem cell populations, able to form differentiated neurons over an extended developmental time. Connection between these two processes might depend on specific changes to regulatory networks, e.g. connections between Hh signal receipt, proliferation and Notch signalling have recently been demonstrated in the chick spinal cord (Saade et al., 2017). However, we can also speculate that quantitative changes may be relevant. Experimental and modelling approaches have shown that the gene regulatory network regulating ventral patterning in jawed vertebrate embryos has intriguing self-organising and scaling properties (reviewed by Briscoe and Small, 2015), and it will be interesting to establish how this might operate in an organism with very low cell numbers and limited cell division such as amphioxus.

An alternative evolutionary scenario would be one of secondary loss of complexity in amphioxus and tunicates. This would imply vertebrate-level complexity was present in the common ancestor of the chordates but has been lost by these animals. We consider the likelihood of this diminished by the evolutionary relationships of these animals, which mean loss of complexity would have to have happened independently in the two lineages. However, the knowledge that the tunicate lineage has indeed undergone a degree of secondary loss, as discussed above, makes it a possibility. Fundamentally however, this is a matter of timing, and as both scenarios pre-date vertebrate origins, it still suggests these evolutionary changes underlie spinal cord cell number and diversity in vertebrates.

MATERIALS AND METHODS

Animal collection and fixation

Naturally spawned *L. planeri* embryos were collected from the New Forest National Park, UK, with permission from the Forestry Commission. Fertilised eggs and embryos were collected by digging at the bottom and surrounding areas of the nests. Embryos were brought to the laboratory and placed in Petri dishes with filtered river water from the same river where they were caught. They were kept at 13-15°C and later fixed at different stages of development following the staging system of Tahara (1988). When necessary, embryos were dechorionated with fine forceps before fixation. Embryos were fixed in 4% phosphate-buffered saline (PBS)-paraformaldehyde (PFA) at pH 7.5, which was cooled on ice before use. Embryos were fixed in an ∼10× excess volume of 4% PFA-PBS with respect to river water at 4°C for at least overnight. After fixation, embryos were washed twice in DEPC-treated 1× PBS for 10 min each, and then dehydrated through a graded series of PBS:methanol (25%, 50% and 75% of methanol in 1×PBS) once for 10 min each. Finally, they were washed twice in 100% methanol for 10 min each and stored in fresh methanol at −20°C. Samples prepared for antibody staining were prepared the same way, except that fixation was restricted to 45 min or 3 h at 4°C.

Cloning and sequence analysis of *L. planeri* genes

Genes were amplified from cDNA from mixed stage *L. planeri* embryos using the following primers: PCNA, forward, 5′-GCACTCGCCAAAATGTTCGA-3′; reverse 5′-ACCGCTGGTCTGTGAAAGTT-3′; Msi2, forward, 5′-ATTC-CCCCGAAGAACACAGC-3′; reverse 5′-GAGACGCGTAGAAGCCGTA-3′; HesB, forward, 5′-CCCCGCTGCCCACGGCAA-3′; reverse, 5′-GCTTT-TTGAGACATTGGCTTTTATTGACATTC-3′; OligA, forward, 5′-GATGA-AGAGCTTGGGCGGAA-3′; reverse, 5′-CTTCATCTCGTCCAGGGAGC-3′. Sequences generated from the amplification have been deposited in GenBank with accession numbers MH020217, MH020218, MH020219 and MH020220. Sequence analysis and manipulation were performed using MAFFT v6.864b (Katoh and Toh, 2008). The parameter for strategy for MAFFT alignments was set as 'auto'. All other parameters were as the defaults. Phylogenetic trees were constructed using MrBayes 3.1, using the mixed model (Ronquist and Huelsenbeck, 2003). One million generations were performed and parallel chains checked for convergence, before discarding the first 25% of trees for calculating consensus trees and posterior probabilities.

DAPT treatments, *in situ* hybridisation and antibody staining

Live embryos were treated with DAPT (N-[N-(3,5-Difluorophenacetyl-L-alanyl)]-S-phenylglycine t-Butyl Ester), a drug that inhibits the Notch signalling pathway. After collecting, embryos were placed in Petri dishes with filtered water from the same river from where they were collected and kept in an incubator at 13-15°C. Embryos that reached developmental stage 24 (Tahara, 1988) were placed in four-well Nunc dishes and 100 µM DAPT (from a 10 mM stock solution dissolved in DMSO) in filtered river water was added. All embryos were allowed to develop until control embryos (treated with the same volume of DMSO) reached stage 26 at 13-15°C. Similar numbers (5-30 per experiment) of control and experimental embryos were treated in parallel. Embryos were fixed in 4% PFA-PBS at 4°C overnight,

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before processing for *in situ* hybridisation. *In situ* hybridisation experiments were carried out as previously described (Lara-Ramírez et al., 2015).

Tubulin staining was conducted with anti-acetylated Tubulin (Sigma T7451) diluted at 1:500 on embryos fixed for 45 min. Embryos were rehydrated in PBS+0.1% Triton X100 (PBT), then blocked overnight at 4°C in 20% heat-treated sheep serum in PBT. Primary antibody was added in block solution and left overnight at 4°C. The next day samples were washed twice for 5 min in PBT, then five times for 1 h in PBT at 4°C before addition of secondary antibody and left overnight at 4°C. To remove secondary antibody, samples were washed twice for 5 min in PBT, then five times for 1 h in PBT at 4°C and imaged. Five to 10 embryos of each condition were included in each *in situ* hybridisation or antibody staining experiment, and experiments were repeated at least twice on different batches of embryos deriving from different fertilisations. Little variation was observed within each replicate, and images reported are representative of the overall group.

For phosphohistone H3 staining embryos were fixed for 3 h on ice in 4% PFA in 1×MOPS-buffered saline (MBS) (pH 7.5) containing 0.1 M MOPS, 1 mM EGTA, 2 mM MgSO4 and 125 mM NaCl. Embryos were washed briefly in 1×MBS and transferred to 15% sucrose in 0.5×MBS for long term storage. Prior to cryo-sectioning, embryos were embedded in Tissue-Tek OCT compound. Serial 12µm transversal sections were collected on Superfrost plus slides, blocked in 10% FBS in Tris buffer (pH 7.5) containing 0.1% Triton X-100 and incubated overnight in anti-pH3 primary antibody (rabbit polyclonal, Millipore, 06-570, 1:1000). A Cy3-conjugated goat anti-rabbit secondary antibody was used and nuclei were stained with DAPI. A total of 70-90 spinal cord sections per embryo were counted for the number of $DAPI^+$ nuclei and $pH3^+$ cells. The proportion of $pH3^+$ cells was calculated as the total number of $pH3^+$ cells observed in the spinal cord divided by the total number of $DAPI^+$ nuclei.

Imaging and image processing

RNA *in situ* hybridised embryos were imaged on a Zeiss Axioskop 2 under DIC optics. Some raw images have been cropped and/or rotated. Images shown in Fig. 4A,B are collages reconstructed by overlaying multiple smaller images of the same embryo, with the plane of focus adjusted between shots to keep the labelled structures in focus. Tubulin antibody stained embryos were imaged on a Leica Mz16F with both control and DAPT-treated embryos imaged under the same illumination conditions. pH3 section images were acquired on a Zeiss 710 confocal microscope. Maximal intensity projections of *z* stacks comprising 6 to 8 1 µm optical sections at 0.6 µm intervals were computed and processed in ImageJ.

Acknowledgements

We thank the Forestry Commission of England for permission to collect lamprey embryos, Vasileios Papadogiannis for assistance with confocal microscopy, and Dr Tatjiana Sauka-Spengler for access to the unpublished *P. marinus* sequence and Notch gene expression data.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: R.L.-R., S.M.S.; Methodology: R.L.-R., C.P.-G., C.P., S.M.S.; Investigation: R.L.-R., C.P.G., C.A., C.P., S.M.S.; Writing - original draft: R.L.-R., C.P., S.M.S.; Writing - review & editing: R.L.-R., C.P.G., C.A., C.P., S.M.S.; Supervision: R.L.-R.; Project administration: R.L.-R.; Funding acquisition: R.L.-R., C.P.-G., C.A., C.P.

Funding

R.L.-R. was supported by the Consejo Nacional de Ciencia y Tecnología (CONACYT). C.P. was supported by a Newton International Fellowship from the Royal Society and by an European Molecular Biology Organization Long-Term Fellowship. C.P.-G. and C.A. were supported by the Erasmus+ programme.

Data availability

New sequences reported in this manuscript have been deposited in GenBank under accession numbers MH020217, MH020218, MH020219 and MH020220.

Supplementary information

Supplementary information available online at
http://dev.biologists.org/lookup/doi/10.1242/dev.166595.supplemental

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