Step-wise evolution of neural patterning by Hedgehog signalling in chordates

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Abstract

In vertebrate embryos Hedgehog (Hh) is expressed by notochord and floorplate cells, and ventral neural cells are patterned by the activities of Hh-regulated transcription factors. Hh signalling is antagonised by signals from the dorsal neural tube, and loss of Hh leads to loss of ventral patterning in the neural tube as the dorsal pattern expands. These mechanisms are critical for producing the neurons that implement motor responses to sensory inputs, but understanding how they evolved has been hindered by lack of insight from commonly-studied invertebrates where nervous system morphology and genetic mechanisms are not conserved with those of vertebrates. The invertebrate chordate amphioxus, which expresses *Hh* in its notochord and floorplate, provides a window into the pre-vertebrate condition. We have examined amphioxus neural development by manipulating function of Hh and downstream genes involved in neural pattern and cell identity. We show that Hh signalling regulates the differentiation of some neurons in amphioxus, including a subset of motor neurons. This demonstrates some conservation of mechanism between vertebrates and amphioxus. However other aspects of neural patterning differ between the two lineages, with amphioxus lacking aspects important in vertebrates. We suggest the complexity of Hh-dependent neural patterning in vertebrates evolved in a step-wise manner. Initial recruitment of Hh occurred in an ancestor to the chordates to regulate the differentiation of a subset of neurons. This was followed, in the vertebrate lineage, by additional changes to the gene regulatory network downstream of Hh, which gave Hh a broader role in dorsal-ventral neural patterning.

Key words: amphioxus; Hedgehog signalling; neural tube patterning; evolution

Introduction

The role of the Hh gene *Shh* in patterning the vertebrate spinal cord is a case study for how a morphogen can control downstream genes to generate patterns of differentiation across a field of cells^{1,2}. Shh from the notochord and floorplate³ drives a gene regulatory network of transcription factors, including members of the Nkx2, Nkx6, Olig and Pax gene families⁴. This causes cells to adopt one of a number of mutually exclusive states, leading to the formation of Dorsal-Ventral (DV) progenitor zones where proliferative neural progenitors lie in the ventricular region. Cells in each zone share a distinct transcriptional profile, and produce daughter cells that move laterally and differentiate into a restricted range of cell types dictated by the zone in which they were born and the time of their birth^{1,2,4}. Ventral patterning by Hh is opposed by dorsal signals from the roof plate, which partition the dorsal neural tube into analogous dorsal progenitor zones^{2,5}. Hh signalling represses dorsal identity, with loss of Hh leading to expansion of dorsal character into the ventral neural tube⁶.

This mechanism is fundamentally the same across vertebrates and its properties have important implications for evolution. First, it has the intrinsic capacity to scale across neural tubes of different sizes, meaning different vertebrate species can use the same mechanism to pattern nervous systems of very different sizes^{7,8}. Second, it generates the high number and diversity of neural cells characteristic of the vertebrate subphylum^{2,9}. This includes motor neurons and sensory neurons, as well as the many populations of interneurons that make connections within the spinal cord and between spinal cord and brain. The number and diversity of these cells lies behind the behavioural sophistication of motor control in vertebrates. Together with innovations in the head in sensory processing and feeding¹⁰, these evolutionary changes underlay the transition of our vertebrate ancestors from relatively passive filter feeders to active predators¹¹.

Understanding how evolution has resulted in such a robust yet adaptable patterning system is a substantial challenge. While some basic aspects of neural development and cell differentiation may be deeply conserved across animals¹², only vertebrates and other chordates form a homologous neural tube with a Hh-expressing floorplate¹³. Invertebrate protostomes like *Drosophila melanogaster* and *Caenorhabditis elegans* lack a comparable role for Hh signalling in neural patterning¹⁴. From an evolutionary and phylogenetic perspective amphioxus is best-placed to address such questions further¹⁵. Amphioxus embryos develop a neural tube and floorplate that are homologous to those of vertebrates, but lack some of their genetic and developmental complexity¹⁶. Historically, however, investigations in amphioxus have been hindered by a lack of methods for precisely manipulating genes.

79 To address this we have investigated the role of Hh in neural patterning in amphioxus using
80 targeted gene knockouts to test the function of Hh signalling and its potential downstream
81 target genes. Through analysis of *Hh*, *Patched (Ptch)*, *OligA*, *Mnxa* and *Err* mutant embryos,
82 coupled with overexpression and rescue experiments, we show Hh signalling does regulate
83 neural patterning through a regulatory network conserved with vertebrates. However, we
84 also identify differences in mechanism. These show that the mechanism we see in living
85 vertebrates evolved in discrete steps: Hh was first recruited to the floor plate of an ancestral
86 chordate to regulate the differentiation of a subset of neurons. In the vertebrate lineage this
87 was elaborated through new regulatory connections to yield the more general patterning
88 mechanism we see in living vertebrates.

Results

Amphioxus *Hh* has been previously described as expressed in the floor plate, but its function here is unknown¹⁷. We first used TALEN-generated *Hh*^{-/-} embryos¹⁸ to ask if neural cells were receiving Hh signals. TALEN lines are maintained as heterozygotes, with crosses producing the genotypes +/+, +/- and -/- in a 1:2:1 ratio (though deviations from this may be encountered when small numbers of embryos are analysed). We processed embryos of these genotypes under identical experimental conditions, allowing comparison of gene expression levels between genotypes within an experiment. A consistent feature of Hh signalling across the Bilateria is that receipt of Hh signal upregulates the expression of the Hh receptor, *Ptch*, while loss of Hh signal reduces *Ptch* expression in neighbouring cells (Fig. 1A)¹⁹. Expression of *Ptch* was correspondingly depressed in *Hh*^{-/-} embryos (Fig. 1B, B'). We noted *Ptch* expression was not completely lost in *Hh*^{-/-} embryos at the neurula stage (Fig. 1B, B'). To establish whether this reflected residual early developmental Hh-independent *Ptch* expression, and that the *Hh*^{-/-} line was indeed a full loss of function allele, we followed *Ptch* expression later in development in *Hh*^{-/-} embryos, and also examined the expression of *Ptch* in *Smoothened* (*Smo*)^{-/-} embryos and LDE225 (a Smo inhibitor) treated embryos, which should prevent cells from receiving Hh signalling and hence resemble the *Hh*^{-/-} phenotype. This confirmed full loss of *Ptch* expression in *Hh*^{-/-} embryos later in development, and showed that the time course of *Ptch* expression in *Smo*^{-/-} embryos matched that in *Hh*^{-/-} embryos. (Supplementary Fig. 1). However, probably due to a low solubility of LDE225 in sea water, *Ptch* expression was only decreased but not abolished in LDE225-treated embryos later in development, even at a saturated concentration (80 µM) (Supplementary Fig. 2).

Conserved aspects of the Hh-Ptch circuitry also predict that loss of *Ptch* function should lead to upregulation of *Ptch* gene expression. We expect a TALEN mutant gene to still be detected by in situ hybridisation in homozygous knockout (-/-) embryos as TALENs produce small deletions with shifted or truncated open reading frames but which should still be transcribed. We therefore examined *Ptch* expression in *Ptch*^{-/-} embryos¹⁸. Correspondingly, in *Ptch*^{-/-} embryos *Ptch* expression was upregulated (Fig. 1J, J'). These data show Hh signals regulate gene expression in amphioxus neural tube cells, and that the pathway behaves as expected from the way it functions in other animals.

We next asked whether gene expression across the neural tube DV axis was perturbed in *Hh*^{-/-} or *Ptch*^{-/-} embryos. In vertebrate embryos many progenitor zone transcription factor genes fall into opposing pairs that cross-repress each other's expression. Ventral genes may be upregulated by Hh signals and hence their expression is reduced or lost when Hh signals

are compromised, while corresponding intermediate and dorsal genes are repressed by Hh and tend to expand ventrally under these conditions. Previous work has examined many of these genes in amphioxus, showing several that have similarly restricted DV expression (²⁰ and references therein). We examined the expression of ten of these genes in *Hh*^{-/-} and *Ptch*^{-/-} embryos. We found that the expression of genes marking the dorsal (*Dlx*, *Pax3/7*) or intermediate (*Pax4/6*, *IrxA*, *IrxB*, *IrxC*) neural tube was not affected in either mutant, with the exception of a small change to *Pax3/7* expression in the brain in relatively late stage embryos (Fig. 1C-D', K-L'; Supplementary Fig. 3). Expression of *Nkx2.2* was not affected, though this gene is only expressed in the ventral portion of the brain and not through the remainder of the neural tube (Supplementary Fig. 3). However, in *Hh*^{-/-} embryos expression of ventral markers *OligA* and *Nkx6* was reduced, as was the floorplate marker *Netrin*, and in *Ptch*^{-/-} embryos *OligA* and *Netrin* expression increased though *Nkx6* was not affected (Fig. 1E-I', M-Q'). More specifically, most *Nkx6* expressing cells were lost (Fig. 1G-H') in *Hh*^{-/-} embryos, while expression of *OligA* was lost in the anterior neural tube, but maintained in the brain, and in more posterior parts of the nervous system (Fig. 1E-F'). These results show some aspects of gene regulation downstream of Hh are conserved between amphioxus and vertebrates, but some are not. Specifically, Hh regulates some ventral neural genes like *OligA* and *Netrin* in both lineages, but more dorsal genes are not affected by Hh signalling in amphioxus. Regulation may also be different in the brain, where *Pax3/7* is regulated by Hh but *Nkx2.2* is not.

In normal amphioxus embryos *OligA* is expressed by reiterated but discrete cells along the Anterior-Posterior (AP) axis (Fig. 1E). In vertebrates, expression of vertebrate *Olig2* and *Nkx6.1* defines the Hh-specified progenitor zone^{1,2} from which motor neurons develop (known as pMN). In many bilaterians (including vertebrates) the motor neuron lineage is marked by the expression of *Olig* and *Nkx6*, plus by the expression of *Mnx*, *Lhx3* and *Islet*^{2,21}. Amphioxus motor neurons have been mapped and fall into three types: dorsal and ventral compartment somatic motor neurons (DCMNs and VCMNs respectively), and visceral motor neurons (VMNs)^{22,23}. These cells use acetylcholine as a neurotransmitter like vertebrate motor neurons²⁴. DCMNs are found in a restricted anterior region of the neural tube and support slow swimming, while VCMNs are found along the length of the neural tube and support rapid swimming²². Most VMNs develop later in development, after the developmental stages studied here. The organisation of *OligA*-expressing cells in amphioxus resembles that of motor neurons. We first used two-colour fluorescent in situ hybridisation to show that several cells co-expressed *OligA* and *Nkx6* (Fig. 2A-C', D). We then examined the expression on *Mnxa*, *Lhx3* and *Islet*²⁵⁻²⁷ in wild type, and *Hh*^{-/-} embryos (Fig. 3 and Supplementary Fig. 4). This showed *Mnxa* was co-expressed by some of these cells in wild

type embryos (Fig. 3 and Supplementary Fig. S). Furthermore, in *Hh*^{-/-} embryos expression of all three genes was reduced or lost by some or all of these cells (Fig. 3C1-E5'). This shows the motor neuron differentiation pathway operates downstream of Hh signaling in amphioxus. In most instances not all neural cells expressing these genes were affected in *Hh*^{-/-} embryos (Fig. 3C1-E5'). This demonstrates both Hh-dependent and Hh-independent aspects of their regulation in different neural cell populations.

To examine further the regulatory relationships between these genes we attempted to rescue *Hh*^{-/-} mutants by mRNA injection. Injection of *Hh*^{-/-} embryos with *Hh* mRNA was able to rescue the expression of *Nkx6*, *OligA*, *Mnxa* and *Lhx3* (Fig. 4). However injection of *Nkx6*, *OligA* and *Mnxa* mRNAs did not rescue the *Hh*^{-/-} phenotype, either individually or when injected in combination (Supplementary Fig. 5). We also generated *OligA* and *Mnxa* TALEN knockout lines (Supplementary Fig. 6). *OligA*^{-/-} embryos appeared normal (Supplementary Fig. 7), though since two paralogues (*OligB* and *OligC*) are co-expressed with *OligA* and are also regulated by Hh signalling (Supplementary Fig. 8), this may reflect functional redundancy. In *Mnxa*^{-/-} embryos, *Mnxa* expression was lost (Fig. 5A, A'), suggesting this gene may self regulate. In addition *Lhx3* and *Islet* expression were reduced in the neural tube of *Mnxa*^{-/-} embryos (Fig. 5D-E'). *Nkx6* and *OligA* were not affected in *Mnxa*^{-/-} embryos despite all three genes being co-expressed (Fig. 5B-C'). This suggests *OligA* and *Nkx6* lie upstream of *Mnxa* in amphioxus, similar to *Olig2* and *Nkx6.1* which lie upstream of the *Mnx* gene *Hb9* in vertebrates.

These experiments demonstrate that Hh signalling regulates the formation of a subset of neurons marked by the expression of *Nkx6*, *OligA*, *Mnxa*, *Lhx3* and *Islet*. Not all cells expressing these genes are affected by loss of Hh signalling, however, suggesting their combined expression marks multiple cell populations. To track the identity of the cell type concerned, we examined the expression of *Vacht*, *Vglut* and *Vgat*, which are markers of acetylcholinergic, glutamatergic and GABAergic/glycinergic neurons respectively²⁴. Motor neurons are acetylcholinergic, while glutamate, GABA and glycine are used as neurotransmitters by different types of interneurons. We found *Vglut* and *Vgat* expression were unchanged in *Hh*^{-/-} and *Ptch*^{-/-} embryos (Supplementary Fig. 9), indicating Hh signalling does not affect the development of these cell types, despite some of them lying in relatively ventral parts of the neural tube. However we found the number of *Vacht* expressing cells was reduced in *Hh*^{-/-} embryos (Fig. 5F, F'), consistent with our interpretation that the loss of *Nkx6/OligA/Mnxa/Islet/Lhx3* positive cells in *Hh*^{-/-} embryos reflects a loss of motor neurons. These data show that in amphioxus ventral Hh signalling regulates the development of cells that activate expression of the motor neuron transcription factor genes of the *Olig*, *Nkx6*, *Islet*, *Lhx3* and *Mnx* gene families. The mechanism is conserved with vertebrates, where

Shh signalling from the floor plate and notochord regulates the development of the pMN progenitor zone, from which *Mnx/Lhx3/Islet* expressing motor neurons differentiate. A clear difference, however, is that in amphioxus these appear as isolated individual cells which differentiate into *Vacht* positive motor neurons, and not via a progenitor domain as in vertebrates.

We also noted that not all *Vacht*-expressing cells were lost in *Hh*^{-/-} embryos. This indicates the presence of an Hh-independent pool of motor neurons. As already mentioned, amphioxus motor neurons fall into three types, VCMNs, DCMNs and VMNs^{22,23}. Since VMNs form later in development we hypothesised that perhaps in early development, one or other of the remaining types of motor neurons was Hh-regulated. To investigate this further, we focused on the nuclear hormone receptor transcription factor gene *Err*, as this has been previously reported to be a marker of DCMNs²⁸. We found that the number of *Err* expressing cells was reduced in *Hh*^{-/-} embryos and increased in the neural tube of *Ptch*^{-/-} embryos (Fig. 5G-H', J, J'). In *Mnxa*^{-/-} embryos no *Err* expressing cells were lost and instead an additional pair of *Err* expressing cells appeared, despite these embryos losing some *Vacht* expressing cells (Fig. 5K-M'). This prompted us to reconsider the expression and role of *Err*. We first generated an *Err*^{-/-} TALEN knockout (Supplementary Fig. 6) to test if this gene was required for motor neuron development. *Vacht* expression was not affected in these embryos (Fig. 5O, O'), and neither was the expression of *Vglut* or *Vgat* (Fig. 5P-Q'). We further used two-colour in situ hybridisation to show that while the domains of *Mnxa* and *Vacht* expression co-localise to the same cells as expected, *Err* is not expressed by these cells or by other cells expressing *Vacht* (Supplementary Fig. 10). The complementary expression of *Err* and *Mnxa* and the ectopic pair of *Err* expressing cells in *Mnxa*^{-/-} embryos led us to ask if the two genes might work antagonistically. *Mnxa* was not affected in *Err*^{-/-} embryos (Fig. 5N, N'), *Err* was, however, repressed by ectopic expression of *Mnxa* (Fig. 5R-S').

These results show that *Err* is not expressed by motor neurons as previously suggested, but instead lies in a parallel pathway in a different population of neural cells. Importantly, however, these cells are regulated by Hh signalling. This shows that in the amphioxus neural tube Hh signals regulate more than just the formation of motor neurons. However, like the Hh-regulated motor neurons, these cells appear as isolated individual cells.

Discussion

Our study shows that amphioxus Hh signalling is received by neural tube cells and that it regulates the differentiation of a subset of neurons via a conserved gene regulatory network involving *Olig*, *Nkx6*, *Mnx*, *Lhx* and *Islet* gene family members. This regulatory signature is characteristic of motor neurons across bilaterian animals²¹, and *Vacht* expression confirms this identity. Hh also regulates another set of cells marked by the expression of *Err*. We do not know exactly what these cells are as they do not express any of the neurotransmitter marker genes we examined, however we know they are not motor neurons as they do not express *Vacht*, and also that their formation (as assessed by *Err* expression) is repressed by *Mnxa*, which is itself induced by Hh. These results show that several aspects of amphioxus DV neural patterning are conserved with vertebrates including (i) the production of Hh by notochord and floor plate cells and the receipt of Hh signals by neural tube cells, (ii) the differentiation of motor neurons under a conserved Hh-regulated gene network, and (iii) the formation of an additional cell type under Hh regulation. However many other aspects of neural patterning and differentiation are not affected by manipulation of Hh signalling in amphioxus. This includes general markers of more intermediate and dorsal neural identity, none of which showed changes in expression in Hh mutant embryos. In vertebrate embryos some of these (*Pax3*, *Pax7*, *Pax6* and *Irx3*) expand into more ventral cells when Hh signalling is compromised⁶, a consequence of the cross-repressive activities of genes downstream of Hh. We also identified many cells expressing specific transcription factors or neurotransmitter genes that were not affected in *Hh*^{-/-} embryos. This included a subset of *Nkx6* positive cells, *Vglut* and *Vgat* positive neurons, a subpopulation of *Vacht* positive neurons, a subset of cells expressing *Islet* and *Lhx3*, *OligA* expressing cells in the brain and posterior neural tube, and an *Mnxa* expressing region in the anterior.

These data show that while some aspects of neural patterning are conserved between amphioxus and vertebrates, there are also considerable differences (Fig. 6). While some ventral neural gene expression is reduced in *Hh*^{-/-} embryos, loss of Hh signalling does not lead to a general ventral expansion of dorsal pattern, as it does in vertebrates⁶. This shows that the general structure of the gene regulatory network downstream of Hh is different, with amphioxus lacking some of the cross repressive interactions characteristic of vertebrates; indeed the only evidence we found for such repression was the effect of ectopic *Mnxa* on *Err* expression, and that an extra pair of *Err* expressing cells appeared in *Mnxa*^{-/-} embryos. However this does not appear to be reciprocal as *Mnxa* was not affected in *Err*^{-/-} embryos. Related to this, cell differentiation in amphioxus is as scattered individual cells undergoing differentiation programmes, only some of which are Hh regulated. This is in contrast to vertebrates where populations of ventral cell types differentiate from their respective

ventricular progenitor zones, with all the ventral progenitor zones established by interactions between genes upregulated by Hh (known as Class II genes) and genes whose expression expands ventrally when Hh signalling is compromised, and are hence described as repressed by Hh (known as Class I genes).

Based on our findings we propose a two-step process by which Hh signalling was incorporated into ventral neural patterning during the evolution of vertebrates (Fig. 6). Hh does not appear to have been primitively involved in midline neural patterning in the Bilateria, as to date no convincing data have demonstrated such a role in protostomes. In the chordate ancestor Hh was then recruited to the ventral midline to regulate the differentiation of some neural cell types, including motor neurons. However assuming amphioxus is reflective of the ancestral condition, the extent of its involvement at this stage in evolution was relatively limited, with many aspects of neural patterning and cell type differentiation not under Hh regulation. At this point in evolution Hh signalling does also not seem to have been regulating such a complex gene regulatory network as we now see in living vertebrates (Fig. 6).

Ascidian tunicate embryos do express *Hh* genes in the floor plate but have a highly reduced neural tube, with few cells and neurons posterior to the brain²⁹. Moreover, disruption of Hedgehog signaling in ascidian embryos does not affect motoneuron formation³⁰. These data indicate ascidian tunicates have lost aspects of neural patterning, and limits their use in understanding the evolution of these characters. However we can conclude that the basic level of involvement of Hh in neural patterning in the chordate ancestor has been elaborated in the vertebrate lineage. Part of this has been the deployment of additional transcription factor genes downstream of Hh. Our data suggest genes expressed in the intermediate and dorsal neural tube are Hh-repressed in vertebrates but not in amphioxus. Since in vertebrates repression is mediated by cross repression between Class I and Class II genes, the evolution of such interactions may underlie this change in patterning. This period of evolution also coincides with the evolution of a large pool of progenitors in the ventricular zone along the length of the vertebrate neural tube³¹, and it is in these progenitors that these and other Hh-regulated Class I and II genes interact to define the DV progenitor zones from which differentiating cells emerge. Further experiments may reveal which of these interactions might also have evolved in early vertebrates, and more precisely show how changes in Hh-regulated patterning map on to the evolution of vertebrate nervous systems and their nuanced control of behaviour¹¹.

Methods

Animal and embryo cultivation and embryo microinjection

Amphioxus (*Branchiostoma floridae*) were originally acquired from Jr-Kai Yu, and the colony maintained under previously-described conditions^{32,33}. Gametes were obtained using the thermal-shock method (from 20°C to 26°C)³². Microinjection was performed as described previously³⁴. Injection solution was prepared containing 20% glycerol, 5mg/mL Texas Red dextran (Life Technology Co.), and desired concentration of synthesized mRNA (*Hh*, 2.0 µg/µl; *Nkx6*, 1.8 µg/µL; *OligA*, 1.4 µg/µL; *Mnxa*; 0.5 µg/µL).

Mutant generation

Hh and *Ptch* mutants have been described previously^{18,35}. Generations and detections of *OligA*, *Mnxa*, *Err* and *Smo* mutants were conducted with the TALEN method as previously described^{36,37}. TALEN target sites of *OligA*, *Mnxa*, *Smo* and *Err*, and mutation types for each gene used in the study are shown in Supplementary Fig. 6. Primers for amplification of flanking regions of *Hh*, *Ptch*, *OligA*, *Mnxa*, *Err* and *Smo* target sites and their amplicon sizes are listed in Supplementary Table 1.

Pharmacological treatments

Hedgehog signaling pathway was activated with SAG (MedchemExpress Co.). A stock solution of 40 mM was made in DMSO and used at final concentrations of 6 - 11 µM. Hh signaling was inhibited with LDE225 (MedchemExpress Co.). A stock solution of 10 mM was made in DMSO and added to embryos at concentrations of 5 - 80 µM. Embryos were treated with SAG or LDE225 from early gastrula stage and fixed at desired stages for in situ hybridization. Controls were treated with DMSO alone. To confirm that LDE225 inhibits or SAG activates Hh signaling in amphioxus, decreased or increased expression of *Ptch* in LDE225 or SAG treated embryos were used as markers. The 80 µM LDE225 and the 11 µM SAG treatments are close to lethal, and hence the highest concentrations that can be used in this developmental window. We also found that at 80 µM LDE225 was at the limit of solubility in sea water.

Plasmid construct preparation

pGEM-T-Easy and pXT7 constructs containing Hh coding sequence were from a previous study¹⁸. To clone the full-length coding sequences of *Pax3/7*, *Dlx*, *Pax4/6*, *Nkx2.2*, *Netrin*, *Err*, *IrxA*, *IrxB*, *IrxC*, *Nkx6*, *OligA*, *Vacht*, *Vglut*, *Vgat*, *Lhx3*, *Mnxa* and *Islet* genes, and the 3'-UTR sequences of *OligA*, *OligB* and *OligC* genes, into relevant expression vectors, a set of primers (Supplementary Table S2) embedded with different restriction enzyme sites were

synthesized according to the sequence data retrieved from the NCBI database. DNA sequences of each gene were separately PCR-amplified from a gastrula cDNA library and then ligated into a pGEM-T-Easy vector (Promega Co.) and verified by sequence analysis. The coding sequences of *Hh*, *OligA*, *Nkx6* and *Mnxa* were cut off from the pGEM-T-Easy construct and ligated into the expression vector pXT7.

In vitro mRNA synthesis

All plasmid DNAs were prepared using Plasmid Mini Kit (Omega Co.) and linearized with either *Bam*HI (for pXT7-derived constructs) or *Sac*I (for TALEN constructs), extracted by phenol-chloroform and dissolved in RNase-free water. *In vitro* mRNA synthesis was conducted using either T7 (for pXT7-derived constructs) or T3 (for TALEN constructs) using the mMESSAGE mMACHINE kit (Ambion Co.).

Whole-mount *In situ* hybridization

The pGEM-T-derived plasmids containing the above genes were linearized by restriction enzyme digest and used to synthesize a digoxigenin (DIG)- or fluorescein-labeled (Roche Co.) antisense riboprobe with Sp6 or T7 RNA polymerase (Promega, USA). Whole-mount *in situ* hybridization (WISH) with single or double probes was performed essentially as previously described^{38,39}. In brief, embryos were hybridized simultaneously with two probes labeled with DIG and fluorescein and detected with anti-DIG-POD and anti-fluorescein-POD antibodies (Roche Co.). The fluorescent signal was then amplified with the TSA Plus Cyanine 3 & Fluorescein system (PerkinElmer Co.). After staining, embryos were washed with PBS, mounted in 80% glycerol and photographed using an inverted microscope (Olympus Co.), or a Zeiss LSM 780 confocal microscope (Zeiss microsystems, Germany). To image the neural plate and neural tube on an inverted microscope, embryos were mounted with the dorsal side uppermost then the focus adjusted to bring the dorsal structures into focus. These are described in figure legends as ventral views focused on the dorsal side. We were sometimes unable to capture whole fluorescently labelled embryos in a single field of view. In such cases two images were taken of different parts of the embryo and these were then overlapped in a collage. This affects panels A-C of Figure 2 and A-C of Figure S10 and in each case delineation of the constituent images has been marked and explicit reference is made in the figure legend.

Embryo genotyping

Genotyping of live embryos was performed as described in³⁷, and genotyping of embryos fixed with 4% PFA-MOPS-EGTA or analyzed by whole-mount *in situ* hybridization was

364 conducted essentially with the same protocol except that an extra 30-min wash in 500 ml
365 filtered sea water or PBS was added before lysis, to remove the fixative.

366 **Data Availability**

367 All data generated or analyzed during this study are in the main text and Supplementary
368 material.

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Figure 1. Neural tube patterning in *Hh*^{-/-} and *Ptch*^{-/-} amphioxus embryos. Anterior is to the left. The scale bars are 50 μm and apply across the respective rows for B-I. The images are in ventral view focused on the dorsal side, except for I, I', Q and Q', which are in lateral view. 3S, 5S and 14S indicates embryos with 3 somites, 5 somites and 14 somites through neurula stages. (A) A simplified summary of Shh signal transduction in vertebrates in the absence (left) and the presence (right) of Shh. Whole-mount in situ hybridization of *Ptch*, *Pax3/7*, *OligA*, *Nkx6* and

490 *Netrin* in the neural tube of (B-I) $Hh^{+/+}/Hh^{+/-}$, (B'-I') $Hh^{-/-}$, (J-Q) $Ptch^{+/+}/Ptch^{+/-}$ and (J'-Q') $Ptch^{-/-}$
491 embryos. The black arrows in D, D', L and L' mark *Pax3/7* expression in the cerebral vesicle. The red
492 brackets in M and M' mark the equivalent position in the neural tube of $Ptch^{+/+}$, $Ptch^{+/-}$ and $Ptch^{-/-}$
493 embryos. Numbers in the bottom right corner of a panel show the number of times the phenotype
494 depicted was seen, out of the total number of embryos from that genotype analysed.
495

Figure 2. Double fluorescent *in situ* hybridization of *OligA* and *Nkx6* in late neurula and schematic diagram of genes expression changes in mutants.

Whole mounts with anterior to the left, the scale bar on A is 50 μ m and applies to all panels. (A) Merge of (B) *OligA* (red) and (C) *Nkx6* (green) expression in WT amphioxus neural tube at 14S (embryos with 14 somites) stage by lateral views. The white arrows in A, B and C mark the

equivalent expression position in the neural tube opposite to somite 1 to somite 4, one arrow each somite. (A') Merge of (B') *OligA* (red) and (C') *Nkx6* (green) expression in WT amphioxus neural tube by ventral views focused on the dorsal side. The white arrows in A', B' and C' mark the equivalent expression positions in the neural tube opposite to somite 1 to somite 4, one arrow each somite. (D) Comparison of neural tube patterning in WT, *Hh*^{-/-} and *Ptch*^{-/-} amphioxus embryos at the 14S stage. The boxes represent neural tube and the lines outside them indicate boundaries between S (somites) to indicate the relative expression position of different transcription factors in neural tube. Panels A-C are each collages of two separate photographs, with the boundary between the images identified in the figure.

Figure 3. The expression of *Nkx6*, *OligA*, *Mnxa*, *Lhx3*, and *Islet* in the CNS through neurula stages in amphioxus *Hh*^{+/+}, *Hh*^{+/-} and *Hh*^{-/-} embryos. Whole mounts with anterior to the left. The scale bar on A1 and A4 is 25 μ m and applies to embryos and transverse sections respectively. Embryos are in ventral views, focused on the dorsal side, except for C1-C5', D1-D2' and E1-E5', which are in lateral view. 3S, 5S, 11S and 14S indicates embryos with 3 somites, 5 somites, 11 somites and 14 somites through neurula stages. (A1-A5') The expression of *Nkx6* from 3S to 14S stage in *Hh*^{+/+}, *Hh*^{+/-} and *Hh*^{-/-}. (A4) Transverse section at level indicated in A3 (arrow). (B1-B5') The expression of *OligA* from 3S to 14S stage in *Hh*^{+/+}, *Hh*^{+/-} and *Hh*^{-/-}. (B4) Transverse section at level indicated in B3 (arrow). (C1-C5') The expression of *Mnxa* from 3S to 14S stage in *Hh*^{+/+}, *Hh*^{+/-} and *Hh*^{-/-}. The black arrowheads in C3, C3', C5 and C5' mark the equivalent expression position in the neural tube opposite to somite 1 and somite 7 in *Hh*^{+/+}, *Hh*^{+/-} and *Hh*^{-/-}. (C4) Transverse section at level indicated in C3 (arrow). (D1-D5') The expression of *Lhx3* from 3S to 14S stage in *Hh*^{+/+}, *Hh*^{+/-} and *Hh*^{-/-}. The black arrowheads in D3 and D3' mark the equivalent expression position in the neural tube

528 opposite to somite 3 and somite 5 in $Hh^{+/+}$, $Hh^{+/-}$ and $Hh^{-/-}$, and the black arrowheads in D5 and D5'
529 mark the equivalent expression position in the neural tube opposite to somite 5 in $Hh^{+/+}$, $Hh^{+/-}$ and $Hh^{-/-}$.
530 (D4) Transverse section at level indicated in D3 (arrow). (E1-E5') The expression of *Islet* from 3S
531 to 14S stage in $Hh^{+/+}$, $Hh^{+/-}$ and $Hh^{-/-}$. The black arrowheads in E3, E3', E5 and E5' mark the
532 equivalent expression position in the neural tube opposite to somite 1 and somite 4 in $Hh^{+/+}$, $Hh^{+/-}$ and
533 $Hh^{-/-}$. (E4) Transverse section at level indicated in E3 (arrow). Numbers in the bottom right corner of a
534 panel show the number of times the phenotype depicted was seen, out of the total number of embryos
535 from that genotype analysed. nt: neural tube; g: gut; s: somites.

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Figure 4. *Hh* mRNA injection restores expression of motor neuron genes in *Hh*^{-/-} amphioxus. Whole mounts with anterior to the left. The scale bar on A is 50 μ m and applies to all panels. Embryos are in lateral views except for C-D', which are in ventral views focused on the dorsal side. (A) Expression of *Nkx6* in control (*Hh*^{+/+} and *Hh*^{+/-}) and (B) *Hh*^{-/-} embryos. (A') Injection of *Hh* mRNA does not induce ectopic *Nkx6* expression in *Hh*^{+/+} and *Hh*^{+/-} embryo neural tubes, (B') but restores *Nkx6* expression in *Hh*^{-/-} embryo neural tubes, as shown in the red dotted boxes in B and B'. (C) Expression of *OligA* in control (*Hh*^{+/+} and *Hh*^{+/-}) and (D) *Hh*^{-/-} embryos. (C') Injection of *Hh* mRNA induces ectopic *OligA* expression in control (*Hh*^{+/+} and *Hh*^{+/-}) embryo neural tubes, (D') and also restores *OligA* expression in *Hh*^{-/-} neural tubes, as shown in the red dotted boxes in D and D'. (E) Expression of *Mnxa* in control (*Hh*^{+/+} and *Hh*^{+/-}) and (F) *Hh*^{-/-} embryos. (E') Injection of *Hh* mRNA does not induce ectopic *Mnxa* expression in *Hh*^{+/+} and *Hh*^{+/-} embryo neural tubes, (F') but restores *Mnxa* expression in *Hh*^{-/-} embryo neural tubes (arrows). (G) Expression of *Lhx3* in the neural tube of control (*Hh*^{+/+} and *Hh*^{+/-}) and (H) *Hh*^{-/-} embryos. (G') Injection of *Hh* mRNA does not induce ectopic *Lhx3* expression in *Hh*^{+/+} and *Hh*^{+/-} embryo neural tubes, (H') but restores *Lhx3* expression in *Hh*^{-/-} neural tubes (compare boxed regions in H and H'). The black arrows in F and F' mark the equivalent position in the neural tube. Numbers in the bottom right corner of a panel show the number of times the phenotype depicted was seen, out of the total number of embryos from that genotype analysed.

Figure 5. Analysis of cell identity in *Hh*, *Ptch*, *Mnxa* and *Err* mutant embryos.

Whole mounts with anterior to the left. Scale bars are 50 μ m and apply to embryos within the block (A and N) or within the same row (for F-M, R and R'). All the embryos are in ventral view focused on the dorsal side except for E, E', J and J' which are in lateral view. Embryos in J and J' are at larval stage, and all others are at neurula stages. The expression of *Mnxa*, *Nkx6*, *OligA*, *Lhx3* and *Islet* in the neural tube of (A-E) control (*Mnxa*^{+/+} and *Mnxa*^{+/-}) and (A'-E') *Mnxa*^{-/-} embryos. The black arrows in D and D', or E and E' mark the equivalent position in the neural tube opposite to somite 1 to somite 5 in *Mnxa*^{+/+}, *Mnxa*^{+/-} and *Mnxa*^{-/-} embryos, one arrow each somite. The expression of *Vacht* and *Err* in the neural tube of (F, G) control (*Hh*^{+/+} and *Hh*^{+/-}) and (F', G') *Hh*^{-/-} embryos. (H, H') Magnification of the anterior embryos shown in G and G'. The red dotted boxes in H and H' mark the equivalent position in the neural tube, and the signals within and outside the boxes represent *Err* expression in the neurons and somites of *Hh*^{+/+}, *Hh*^{+/-} and *Hh*^{-/-} embryos, respectively. The expression of *Vacht* and *Err* in the neural tube of (I, J) control (*Ptch*^{+/+} and *Ptch*^{+/-}) and (I', J') *Ptch*^{-/-} embryos. The brackets in J and J' mark the equivalent position in the neural tubes of *Ptch*^{+/+}, *Ptch*^{+/-} and *Ptch*^{-/-} embryos. The expression of *Vacht* and *Err* in the neural tube of (K, L) control (*Mnxa*^{+/+} and *Mnxa*^{+/-}) and (K', L') *Mnxa*^{-/-} embryos. Magnifications of the neural tube areas of L and L' are shown in M and M', with the red box outlining an additional pair of cells seen in M'. The expression of *Mnxa*, *Vacht*, *Vglut* and *Vgat* in the neural tube of (N-Q) control (*Err*^{+/+} and *Err*^{+/-}) and (N'-Q') *Err*^{-/-} embryos. The expression

of *Err* in (R) control and (S) *Mnxa* mRNA-injected embryos. (R', S') Magnification of the anterior embryos shown in R and S. The red dotted boxes in R' and S' marks the equivalent position of neural tube, and the signals within and outside the boxes represent *Err* expression in the neurons and somites of control and *Mnxa* mRNA-injected embryos, respectively. Numbers in the bottom right corner of a panel show the number of times the phenotype depicted was seen, out of the total number of embryos from that genotype analysed.