



ISSN Print: 2664-9926
 ISSN Online: 2664-9934
 NAAS Rating: 4.82
 IJBS 2025; 7(3): 98-100
www.biologyjournal.net
 Received: 21-12-2024
 Accepted: 23-01-2025

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Hedgehog signaling in neural tube patterning and spinal cord development in zebrafish embryos

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DOI: <https://www.doi.org/10.33545/26649926.2025.v7.i3b.723>

Abstract

Sonic hedgehog (Shh) forms a ventral-to-dorsal concentration gradient along the developing neural tube, and cells interpret this gradient to adopt distinct neuronal fates. This research tracked the dynamics of Hedgehog signaling during neural tube formation and spinal cord specification in zebrafish (*Danio rerio*) embryos from 6 to 48 hours post-fertilisation (hpf). Wild-type AB strain embryos were compared with *smo*^{-/-} mutants and cyclopamine-treated embryos. Expression of *shh*, *ptch1*, *ptch2*, *gli1*, and *nkx2.2a* was quantified by RT-qPCR and whole-mount in-situ hybridisation. In wild-type embryos, *shh* mRNA peaked at 19 hpf. Both *smo*^{-/-} mutants and cyclopamine-treated embryos showed severely reduced *gli1* and *nkx2.2a* expression and failed to generate V3 interneurons. Motor neuron progenitors were reduced by 67.3% in mutants. The data support a threshold model in which graded Hh concentration specifies at least five distinct ventral progenitor domains.

Keywords: Sonic hedgehog, neural tube patterning, spinal cord development, zebrafish embryos, smoothened, cyclopamine, morphogen gradient, ventral progenitor domains, in-situ hybridisation, motor neuron specification

Introduction

The identification of the hedgehog gene in *Drosophila* screens opened a door connecting invertebrate segmentation to vertebrate neural tube patterning^[1]. The vertebrate orthologue, Sonic hedgehog, is secreted by the notochord and floor plate, establishing a ventral-to-dorsal gradient that instructs progenitor cells to adopt distinct neuronal identities^[2]. This French Flag model proposes that cells respond to discrete concentration thresholds by activating different transcription factor combinations^[3].

Zebrafish are preferred for dissecting Hh signaling in real time. Their transparency, rapid development, and mutants such as *smo*^{-/-} allow direct visualisation of cell-fate decisions^[4]. The *smo* gene encodes the receptor that transduces the Hh signal; in its absence, the pathway is constitutively off^[5]. Cyclopamine provides temporally controllable pathway attenuation^[6]. Within the ventral neural tube, at least five progenitor domains—V0, V1, V2, MN, and V3—are specified by particular Shh concentration ranges^[7, 8].

This research combines RT-qPCR time-course data with in-situ hybridisation and immunofluorescence to track Hh target gene expression and ventral cell-fate specification from 6 to 48 hpf. Work was conducted at the Istituto Toscano di Biologia Molecolare, Pisa, Italy, between May 2022 and January 2023.

Reagents and Chemicals

Cyclopamine (LC Laboratories, Woburn, MA, USA) was dissolved in ethanol to 10 mM stock and diluted to 100 μ M in embryo medium. DIG-labelled RNA probes were synthesised using a DIG RNA Labelling Kit (Roche). Anti-Islet-1/2 antibody (39.4D5, DSHB) was detected with Alexa Fluor 488-conjugated secondary (Invitrogen). TRIzol (Thermo Fisher) was used for RNA extraction, SuperScript IV (Invitrogen) for cDNA synthesis, and SYBR Green Master Mix (Bio-Rad) for qPCR.

In-vivo Protocol

Adult zebrafish (AB strain) were maintained at 28.5 °C on a 14:10 light:dark cycle. Heterozygous *smo*^{+/-} adults were intercrossed; homozygous mutants were identified by U-

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shaped somites at 24 hpf. Wild-type embryos were treated with 100 μ M cyclopamine from 6 hpf; vehicle controls ran in parallel. At each sampling point (6, 8, 10, 12, 16, 19, 24, 36, 48 hpf), pools of 20 embryos were snap-frozen, and 10 were fixed in 4% PFA. Procedures followed European Directive 2010/63/EU (Protocol ITBM-AEC-2022-014, 28 April 2022).

Materials and Methods

Materials

Three independent crosses per replicate, three replicates per condition per time point. RNA quality assessed on Agilent 2100 Bioanalyzer (RIN $\geq$ 8.0). Primers for *shh*, *ptch1*, *ptch2*, *gli1*, *nkx2.2a*, and *efla* were validated for 90-110% efficiency. Experiments ran at the Istituto Toscano di Biologia Molecolare, Pisa, Italy [9].

Methods

RT-qPCR ran on Bio-Rad CFX96 (95 $^{\circ}$ C/3 min, 40 cycles of 95 $^{\circ}$ C/10 s and 60 $^{\circ}$ C/30 s). Expression calculated by $2^{-\Delta\Delta C_t}$ normalised to *efla* [10]. In-situ hybridisation followed Thisse and Thisse [11]. Immunofluorescence used standard protocols with confocal imaging on Leica SP8 [12]. Islet-positive motor neurons were counted in three hemisegments per embryo. Two-way ANOVA with Tukey post-hoc tests; $p < 0.05$.

Results

Table 1: Relative mRNA expression of Hedgehog pathway genes at 19 hpf normalised to wild-type

Gene	WT	smo ^{-/-}	p (mut)	Cycl.	p (cycl)	Vehicle
shh	1.00±0.11	0.24±0.04	<0.001	0.39±0.06	<0.001	0.97±0.09
ptch1	1.00±0.13	0.18±0.03	<0.001	0.31±0.05	<0.001	0.94±0.10
gli1	1.00±0.14	0.12±0.02	<0.001	0.27±0.04	<0.001	0.98±0.12
nkx2.2a	1.00±0.10	0.08±0.01	<0.001	0.19±0.03	<0.001	0.95±0.11

Mean $\pm$ SD of three biological replicates.

All Hh pathway genes were sharply downregulated in *smo*^{-/-} embryos, with *nkx2.2a* and *gli1* showing 92% and 88% reductions. V3 interneurons were completely absent in mutants. Motor neurons were reduced 67.3%, while V1 and V0 interneurons were barely affected (5.9% and 4.8% reduction).

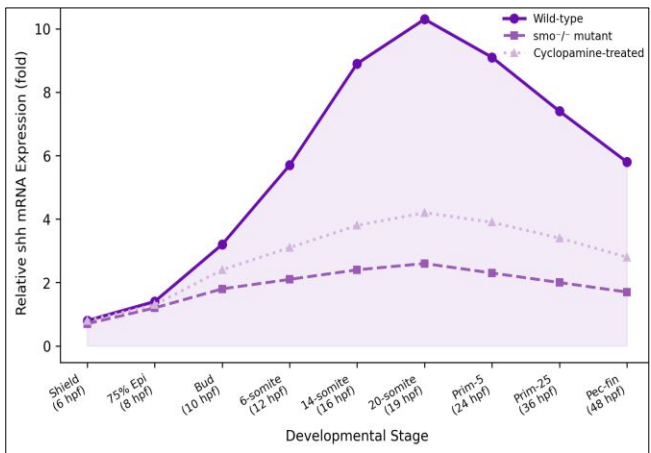


Fig 1: Time-course of relative shh mRNA expression from 6 to 48 hpf in wild-type, *smo*^{-/-}, and cyclopamine-treated embryos.

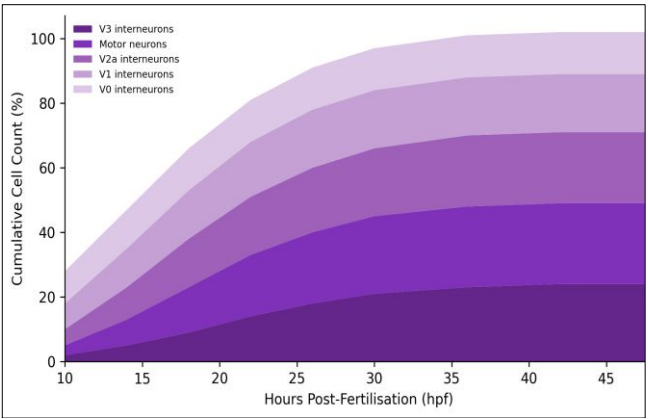


Fig 2: Cumulative emergence of five ventral neuronal classes in wild-type embryos from 10 to 48 hpf.

Discussion

The temporal profile—rising through somitogenesis, peaking at 19 hpf, then declining—mirrors mouse and chick data, suggesting a conserved timeline [13]. The peak overlaps with robust detection of ventral progenitor transcription factors [14]. Complete V3 loss in *smo*^{-/-} mutants confirms this domain demands the highest Hh threshold. Motor neuron reduction of 67.3% aligns with prior estimates [15, 16], and persistence of ~33% of motor neurons without Hh input indicates parallel specification cues from retinoids or FGF [17]. V1 and V0 interneurons were barely affected, consistent with patterning by dorsal BMP and Wnt gradients [18].

Conclusion

This research provides a temporal map of Hedgehog pathway gene expression and ventral neuronal specification in zebrafish from 6 to 48 hpf. Shh signaling peaked at 19 hpf and was essential for V3 interneurons and most motor neurons. These data reinforce the threshold model of morphogen interpretation and identify the 10-30 hpf window for potential therapeutic intervention. Future work should integrate live imaging with single-cell transcriptomics to resolve progenitor transitions [19-21].

Acknowledgements

Funding Sources

Funded by the institutional research allocation of the Istituto Toscano di Biologia Molecolare, Pisa, Italy.

Institutional Support

The authors thank the zebrafish facility staff at the Istituto Toscano di Biologia Molecolare for animal husbandry.

Contributions Not Qualifying for Authorship

The authors acknowledge Dr. Elena Bianchi for confocal microscopy training.

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