Naproxen exposure during early development inhibits cranial chondrogenesis in axolotl embryos

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INTRODUCTION

Naproxen (NPX) is a non-steroidal anti-inflammatory drug (NSAID) commonly used to alleviate pain and inflammation via inhibition of the enzyme cyclooxygenase (COX). *In utero* exposures to NSAIDs have been linked to preterm birth, neural tube closure defects, and orofacial malformations, which may be linke to abnormal neural crest cell (NCC) development. NCCs are stem-like cells that differentiate into numerous adult tissues including craniofacial cartilage and bone and neurons of the peripheral nervous system.

Preliminary experiments in the Rogers Lab:

- Identified COX1 and COX2 transcript and protein expression during the early stages of vertebrate embryonic development
- Showed that targeted knockdown of COX2 and its receptor, EP3, using translation-blocking morpholinos leads to aberrant NCC development in vertebrate embryos

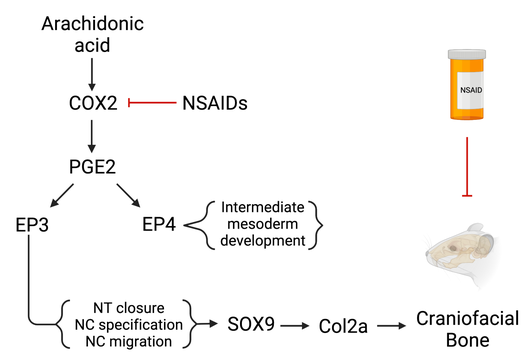


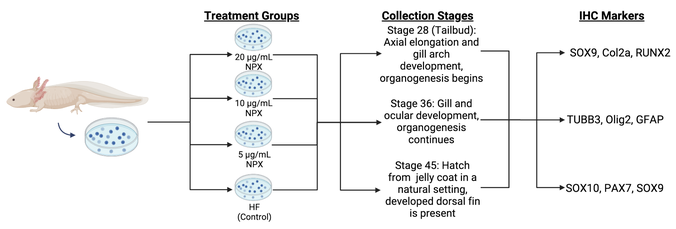
Figure 1: Proposed mechanism for NPX-induced inhibition of cranial chondrogenesis.

Our overall goal is to investigate the molecular mechanisms underlying the development of craniofacial defects following exposure to NSAIDs. We *hypothesized* that exposure to NPX during early development will inhibit cranial cartilage development. To test this hypothesis, we exposed *Ambystoma mexicanum* (axolotl) embryos to various concentrations of NPX during NCC migration and differentiation stages and then performed immunohistochemistry (IHC) for markers of NCC-derived cells. We identified that:

- NPX exposed embryos show decreased survival and exhibit molecular changes at st. 28 and gross anatomic changes by st. 36.
- Exposed embryos have abnormal expression of SOX9 in NCCs.
- SOX9 loss results in abnormal spatial expression of Col2a and absent formation of discrete craniofacial cartilage structures.
- NPX also appears to disrupt normal RUNX2 expression and patterning in putative precursor cells of the lateral line sensory system.

METHODS

Figure 2: Experimental design. Axolotl embryos were divided into 4 treatment groups with corresponding concentrations of NPX in Holtfreter's (HF; control). They were grown to various stages (st. 28, 36, and 45) and collected for IHC using one of the three sets of markers shown.



Marker	Protein Type	Associated Cell Type/Structure
SOX9 SOX10 PAX7	Transcription Factors	Neural Crest
RUNX2		Developing lateral line organs and osteogenic NCCs
Olig2		Oligodendroctye precursors and developin motor neurons
TUBB3	Cytoskeletal Components	Protein component of microtubules in neurons
GFAP		Intermediate filament protein associated with astrocytes and ependymal cells of the CNS
Col2a	Extracellular Matrix-Associated	Exctracellular matrix protein secreted by chondrocytes during cartilage formation and endochondral ossification

Figure 3: Overview of IHC markers used. The markers used in this project can be divided into 3 main groups: neural crest-specific markers (SOX10, PAX7, and SOX9), markers of NCC-derived chondrogenesis (SOX9, Col2a, and RUNX2), and non-chondrogenic NCC-derivative markers (TUBB3, GFAP, and Olig2).

RESULTS

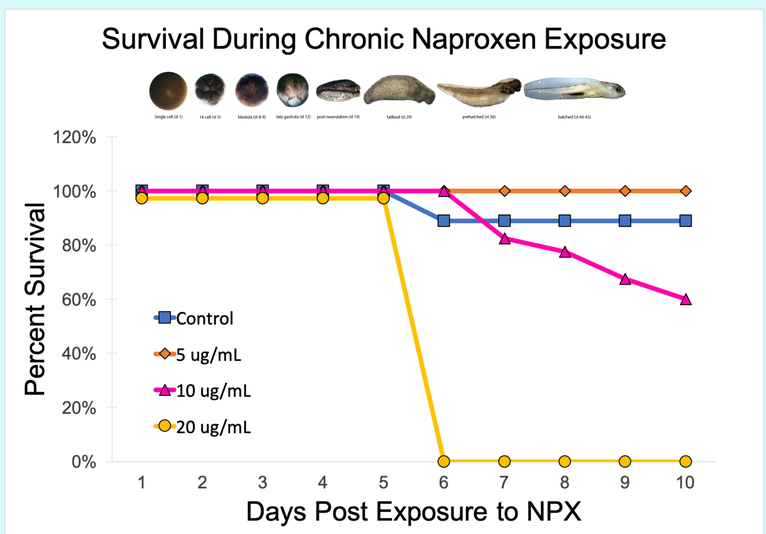


Figure 4: Axolotl embryos exposed to NPX exhibit decreased survival. We identified that the degree of exposure to NPX in axolotl embryos correlates with their rate of survival during development. There appears to be a significant decrease in survival rate between 5 and 7 days post exposure to NPX which corresponds with developmental stages 34-40 and progression of organogenesis.

RESULTS

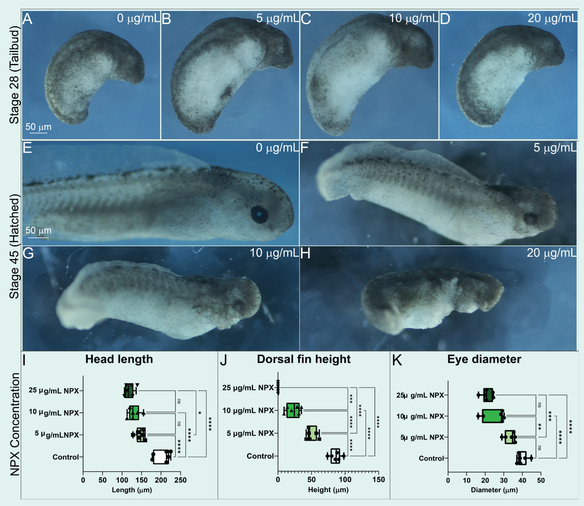


Figure 5: The level of NPX exposure corresponds to the severity of morphologic defects observed in late stage axolotl embryos. (A-D) In st. 28 embryos, the degree of exposure to NPX appeared to have no effect on cranial morphogenesis demonstrated by the lack of observable defects. (E-H) However, in st. 45 embryos, increasing concentration of NPX corresponded with the development of progressively more severe morphological defects. (I-K) When compared to the control, all 3 treatment concentrations produced statistically significant decreases in head length, dorsal fin height, and eye diameter in st. 45 embryos.

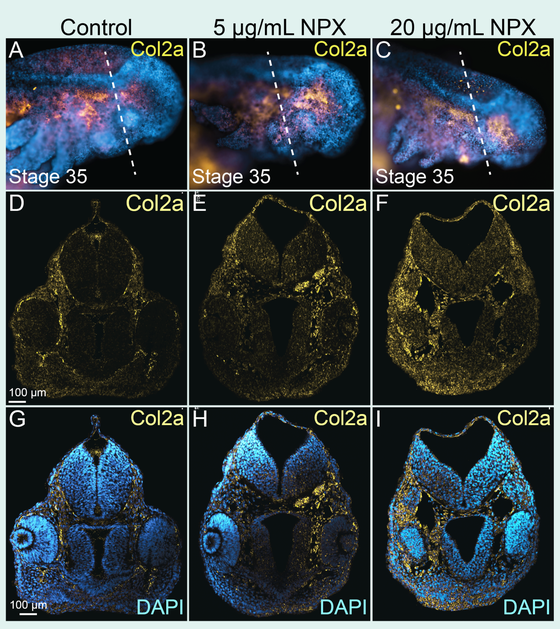


Figure 6: Exposure to NPX causes abnormal distribution of Col2a within the developing head. IHC for Col2a in (A-C) whole mount and (D-I) transverse sections of st. 36 axolotls. When concentration of NPX was increased, Col2a expression was abnormally localized within the developing head corresponding to a lack of proper migration.

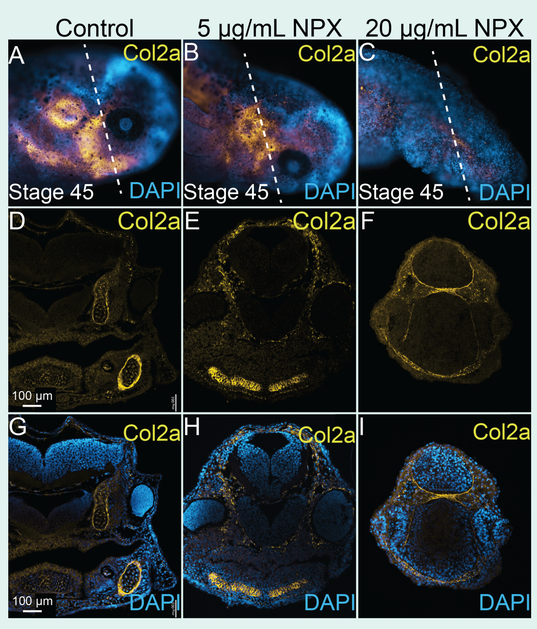


Figure 7: Abnormal Col2a expression causes abnormal formation of cartilage structures at late stages. IHC for Col2a in (A-C) whole mount and (D-I) transverse sections of st. 45 axolotls. We observed significantly decreased organization of cartilage and Col2a-expressing cells in treated embryos to form the discrete structures present in control embryos.

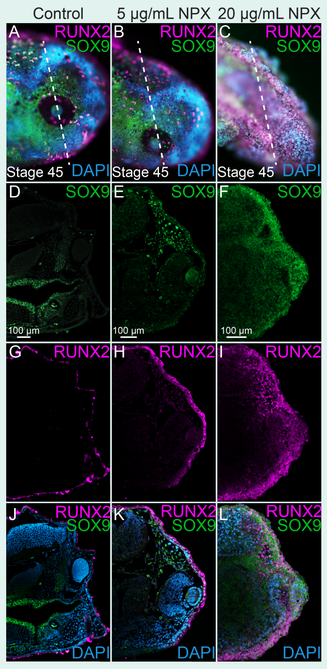


Figure 8: NPX alters RUNX2 expression and patterning in the cranial region of developing axolotl embryos. IHC for RUNX2 and SOX9 in (A-C) whole mount and (D-L) transverse sections. RUNX2 expression appears upregulated in treated embryos and exhibits abnormal patterning including absence of discrete polarized cellular packets of RUNX2 epidermis.

SUMMARY

- Axolotl embryos exposed to NPX exhibit decreased survival.
- In late stage embryos, the level of NPX exposure corresponds to the severity of morphologic defects that occur.
- Exposure to NPX causes abnormal spatial localization of Col2a within the developing head of axolotl embryos.
- NPX inhibits formation of discrete craniofacial cartilage structures necessary for craniofacial bone development in late stage embryos.
- RUNX2 expression is upregulated following exposure of embryos to NPX.
- Normal patterning of RUNX2 expression in putative lateral line sensory system is lost due to NPX exposure.

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