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JOURNAL OF EVOLUTIONARY SCIENCE
ISSN NO: 2689-4602

Review

DOI: 10.14302/issn.2689-4602.jes-21-3868

Evolutionary Conservation of Hox Genes in Vertebrate Brain Development

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Abstract

Hox genes, their conserved derivatives, and the pathways responsible for their expression have been extensively studied in the fruit fly, *Drosophila melanogaster*; the experimentation done in the *Drosophila* model system has given developmental biologists tools to better understand the role and significance of *Hox* genes and their derivatives in anterior-posterior axis determination in the *Drosophila* embryo. Along with this, *Drosophila* research opened up the door to investigation on the conservation of *Hox* genes between vertebrates and invertebrates. Comparative embryology in mice, chickens, pufferfish, and zebrafish have shown conserved *Hox* gene expression patterns specifically along the anterior-posterior axis. Recently, comparative analysis performed on dorsal-ventral axis formation showed that patterning and segmentation of the spinal cord is influenced by the action of *Hox* genes as well. This review will briefly consider the evolution of the vertebrate brain and the evolution and conservation of *Hox* genes in regulating hindbrain patterning and spinal cord development.

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Keywords: *Hox* genes, hindbrain segmentation, duplication and divergence, evolutionary conservation, rhombomeres

Received: Jun 04, 2021 **Accepted:** Jun 10, 2021 **Published:** Jun 12, 2021

Editor: Jianliang Jin, Department of Anatomy, School of Basic Medicine, Nanjing Medical University

Introduction to the Brain and Nervous System

The brain is a complex and highly organized structure showing extremely high levels of patterning, segmentation, and symmetry[1]. Symmetry and asymmetry amongst organisms are what distinguishes and classifies them into morphological categories; the brain is no exception [2]. The second largest region of the brain is the cerebrum composed of the left and right hemispheres. The functional asymmetry of the cerebrum's activity is seen in how these two halves control the body; the left hemisphere dictates the behavior of the right half of the body while the right hemisphere dictates the functions of the left half of the body. Contrasted to the cerebrum, the cerebellum, the symmetrical portion of the brain under the brainstem, is the second largest part of the brain and is solely responsible for maintaining balance, control of movement, and coordination[3]. The conservation of symmetry and asymmetry observed in invertebrate brains and vertebrate brains allows inferences about the conservation of the mechanisms that pattern the brain in early embryonic development. Examination of left-right patterning mechanisms in the brains of vertebrates and invertebrates reveals that these asymmetrical properties are the result of the highly conserved *Nodal* cascade, which is responsible for morphogenesis, and placement of the nervous system organs [4].

It is valid to assume that if one of the most complex processes that pattern the symmetry and asymmetry of our brains has been conserved over millions of years, other processes have also been conserved over time. Similarly, does the vertebrate brain, and in this context, the hindbrain and spinal cord of the mammalian brain, possess a shared derived characteristic for development, segmentation, and neuronal formation with other vertebrates and invertebrates? Evolutionary connectomics of the mammalian neocortex reveals that the mammalian brain is not as unique as previously supposed; Karten and colleagues draw attention in their review to Charles Darwin's *Origin of the Species* (1859) and how his work stimulated early neurobiologists to realized that the theory of natural selection affects the conservation of genes resulting in shared derived characteristics among mammalian and non-mammalian brains[5]. Darwin indicated that the process of natural selection is "highly conserved and cumulative" and does not necessarily result in striking anatomical differences over time. This concept further supports the hypothesis that even minute variations in the genome could affect the conservation of genes over time and subsequently, evolve into shared derived characteristics [5]. The novel idea that molecular pathways in mammalian brain development are not unique to mammals was not well accepted in the field of neurobiology. Subsequent research on the conservation of brain development prompted the discovery of *Hox* genes and their derivatives, which are primarily responsible for the shared derived characteristics observed today in the embryonic nervous system of all vertebrates.

Overview of Hox Genes

Hox genes are master regulatory genes that share a highly conserved DNA sequence called the homeobox. The eight *Hox* gene paralogues present in *Drosophila,* called the homeotic complex, were identified through mutations of the thorax, abdomen, legs, and antennae that caused homeotic transformations [6]. Over time, more research emerged on these master regulatory genes showing their evolution and conservation among organisms. In *Drosophila,* the *HOM-C* complex*,* which is responsible for the body patterning, segmentation, and positional specification along the anterior-posterior axis in the *Drosophila* embryo, has conserved homologues in vertebrates, which are expressed in the same pattern of colinearity that is characteristic of *Hox* gene expression in the *Drosophila* embryo[7]. The homeotic selector genes *antennapedia (Antp)* and *bithorax* are highly conserved and possess derivatives that can be found in paralogous groups on four different chromosomes of the vertebrate genome. The chromosomes are labeled with letters A-D and the paralog groups are numbered from 1-13 with each group containing 9-11 different *Hox* genes[6]. Vertebrate *Hox* gene clusters contain the instructions for anterior-posterior and dorsal-ventral axis patterning among vertebrates, which is similar to the *HOM-C* complex that segments and patterns the body of the fruit fly, *Drosophila melanogaster,* and other invertebrates.

Conservation of Hox Genes

Lappin *et al.* [7] reviewed the evolution of *Hox* genes and found that their presence could be mapped back to a common ancestor of plants around 1,000 million years ago. These organisms phenotypically do not possess segmentation, which was prior to this finding, a primary indicator of ancestral homeobox gene possession given that *Hox* genes are responsible for body patterning and segmentation. They found that plants, fungi, mollusks, echinoderms, and urochordates also possessed conserved forms of homeobox genes. Observation and analysis done on the 13 groups of *Hox* genes in vertebrates point to the conclusion that groups 1-4 pattern the developing hindbrain and groups 5-13 pattern the developing the spinal cord [8, 9]. Viewing these data from a molecular evolutionary perspective, this deviation could be due to numerous whole genome duplications over time. This idea is termed the duplication and divergence model, or the duplication-first model [10]. *Hox* genes have been highly conserved throughout metazoan evolution, from phylum Cnidaria, class Anthozoa (Sea Anemone) to phylum Chordata, order Primates (Humans). This means that the same genes responsible for segmentation and regionalization in the organismal body plan invertebrates (e.g. *Drosophila melanogaster*) are still responsible for the specification and determination of parts of the vertebrate body plan as well. Moreover, evidence indicates that the somites involved in body patterning and segmentation are not the primary regions in which *Hox* genes are expressed in the vertebrate body plan; rather, it is the nervous system in both *Drosophila* and vertebrates [6].

The Central Nervous System and Hox Genes

The central nervous system (CNS) is polarized at the earliest stage of brain development in vertebrates and invertebrates. The mechanism by which embryonic ectoderm is polarized to neuroectoderm is a result of a conserved molecular signaling system known as the Decapentaplegic (Dpp)/Bone morphogenetic protein (BMP) signaling cascade [11]. BMPs, such as *Noggin* and *Chordin,* are essential for the formation and development of the central nervous system (CNS) because they activate the signaling pathway responsible for the differentiation of the non-neural ectoderm into the neural plate. The morphogens released from the signaling cascade produces an antineurogenic effect which represses the identity of the neurons to the dorsal side of vertebrates – mice, chickens, zebrafish - and the ventral side of invertebrates – fruit flies and tunicates. This repressive antineurogenic effect localizes the formation of the neuroectoderm to the respective dorsal or ventral side of the embryo that does not express Dpp/ Bmp. Pro-neural genes found in *Drosophila* – *achete, scute,* and *lethal of scute* – have homologues in mammals, which are also pro-neural genes that are necessary for the generation of neuronal tissue and neural stem cells. These homologues include *Mash1, Ngn1,* and *Ngn2* [11, 12]. Additionally, another ancestral gene found in the *Drosophila* embryo – *achaete-scute* – is involved in the control of sensory neurons in *Drosophila* and is controlled by the homeobox gene, *Ubx,* which is homologous in function and composition to *Mash1* in vertebrates. Specifically, in mammals, the orthologue *Mash1* has a positive effect on the formation of noradrenergic interneurons; *Ubx* has the opposite effect in *Drosophila* given that it suppresses this action along with the formation of bristles in the bithorax. Nonetheless, these two orthologues act in a similar fashion concerning the specific stage of development in which they are activated [10, 13]. Subsequent analysis [11] revealed another gene family that is also highly conserved from invertebrates to vertebrates known as the *ems/Emx*, which is necessary for early patterning and segmentation of the brain and spinal cord in both invertebrates and vertebrates.

Experimental analysis [10, 11] demonstrated that the Notch signaling pathway has been highly conserved over time and is essential in both vertebrates and invertebrates for the formation of the neuroepithelium. Despite the identification of these pathways [10, 11, 12, 13], there still seems to be a gap in the evolutionary conservation of the genes responsible for head and brain development among invertebrates and vertebrates. Closer analysis of this conservation and the gap that seems to persist between invertebrates and vertebrates was performed to bring clarity to the genetic and molecular similarities that humans share with other vertebrates and insects. This research [10] focused mainly on cartilaginous fish and cyclostomes because these phyla are the most promising model system for closing

the "gap" of *Hox* gene conservation among invertebrates and vertebrates. In cartilaginous fishes, *Hox* genes were found to have well conserved ancestral cluster organization. The first evidence of this cluster organization came from the horn shark, which is the most recently evolved member available for experimentation. The only deviation in cartilaginous fishes in terms of the *Hox* genes is the absence of HoxC in the spotted dogfish. Otherwise, the entire genome sequence of *Hox* genes and their derivatives in cartilaginous fishes conserved more ancestral members of *Hox* genes than the class of bony fishes (Osteichthyes). Furthermore, intergenic sequences within the Hox clusters themselves have been conserved over a period of 250 million years [10].

Gap Gene Conservation in Invertebrates and Vertebrates

The genetic pathways that act in tandem with *Hox* genes have also been conserved across phyla. For example, Reichert and colleagues [11] showed that the cephalic Gap gene *otd/Otx*, which is essential for the formation of the anterior brain primordium and for the marking of the anterior portions of the embryo up to the midbrain/hindbrain boundary, is conserved in insects and mice and is responsible for the growth and development of the anterior brain primordium. *Otx1* and *Otx2* are mouse orthologues of the *Drosophila* cephalic Gap genes; in both *Drosophila* and mouse embryos, when the *otd/Otx* Gap gene was knocked out, the premature anterior portions of the brain did not form properly, thus concluding that this cephalic Gap gene is necessary of the formation of the anterior brain primordium. *Otx* orthologue expression has also been discovered in human, chick, *Xenopus,* and zebrafish embryos, supporting the adaptation and conservation of Gap genes across metazoans [14]. Even before the formation of neuronal structures, the pathway responsible for the generation of the neuroectoderm was also highly conserved among animals [11, 15]. Conservation of this cluster organization and positioning is also seen in the lamprey; it contains a similar pattern of expression and *Hox* gene cluster organization in comparison to mammals. The only difference between the two is that mammals possess four *Hox* clusters while lampreys possess six [16]. The most parsimonious explanation for the conservation of clusters and possession of excess clusters among vertebrates is the duplication and divergence model.

Hox Gene Evolution through the Duplication and Divergence Model

The duplication and divergence model of evolution states that the highly conserved *Hox* genes seen in many different species have duplicated before each one of the lineages diverged overtime from a common ancestor [9]. This hypothesis is supported by observations of *Hox* genes in amphioxus (i.e. lancelet; phylum Chordata, subphylum Cephalochordata). Amphioxus does possess ancestral characteristics of chordate anatomy but lacks cranial neurons and neural-crest derived mesenchyme, the primary region where *Hox* genes act in chordates and more specifically, vertebrates. It is interesting though that these organisms possess somite-like blocks in their spinal cord indicating that *Hox* genes may play a part in the development of amphioxus. The conclusions of this analysis showed that the amphioxus *Hox* clusters observed shared many similarities to mouse *Hoxb-3* proteins and sequence organization pointing to homologies among vertebrate *Hox* clusters and amphioxus *Hox* clusters, further supporting the duplication and divergence model of *Hox* genes [8].

Research done on zebrafish and other teleost *HoxD* complexes revealed that the majority of teleosts possess all four ancestral clusters of mammalian *Hox* genes. In zebrafish specifically, the entire 5' end of the *HoxD* cluster found in mammals is preserved in zebrafish [17]. The genomic data of hagfish (clade Cyclostomata, Myxini) was also examined and nineteen *Hox* genes were detected. Genes from paralogous group 1 (PG1) which are significant in rhombomere, reticulospinal, and branchiomotor neuron specification were also detected in the hagfish [18]. Taken together these data reveal that fibrous skeleton fishes (e.g. hagfish) cartilaginous fishes (clade Chondricthyes, Elasmobranchii: sharks and rays), and ray-finned fishes (clade Osteichthyes, clade Teleost, class Actinopterygii: zebrafish) possess extremely well conserved ancestral clusters of *Hox* genes [17, 19]. Conclusions from the data obtained in these studies on the duplication and divergence model point to the idea that even if genes are duplicated, the resulting duplicates could amass

numerous deleterious mutations, become a pseudogene, or be completely lost due to independent gene loss in a lineage [17, 20]. Nonetheless, these genes could still prove to be essential to development and overall function of the vertebrate embryo. This hypothesis was supported when viewing the duplicated genes of *Hoxb1* in zebrafish; both *hoxb1a* and *hoxb1b* were found to be necessary for specification of segments in the hindbrain, termed rhombomeres, thus supporting the hypothesis of sub-functionalization [20]. Experiments performed on pufferfish, which have fused bones in the head and jaw and lack ribs and pelvic fins, were also found to have duplicated genes that were necessary for development and specification in a more "primitive" vertebral structure. Both pufferfish and zebrafish share *Hoxab, Hoxaa, Hoxba,* and *Hoxbb* clusters; the difference among these four duplicates is roughly zero to two genes. This conservation supports duplication before the divergence of the pufferfish and zebrafish lineages. Another example of the duplication/divergence model is the gene *Hoxa7a*, a pseudogene in the pufferfish lineage; *Hoxa7a* is functional in fellow clade members' striped bass and tilapia being necessary for proper skeletal development [21]. These data show that this gene is necessary for development in striped bass and tilapia but not in pufferfish. This is most likely due to inactivity of the pseudogene in pufferfish given that it had a specified function in closely related species. One conclusion is that given the persistence of *Hox* gene clusters, the lack of segmentation in the body plan of an organism does not discourage the expression and apparent necessity of these genes for development. Examination of *Hox* genes and their specific roles in the development of the anterior-posterior (A-P) and dorsal-ventral (D-V) axes in vertebrates and invertebrates has been rapidly emerging since the discovery of *Hox* genes.

The common theme among the majority of this research is that *Hox* genes do not pattern the body of vertebrates like they do in *Drosophila;* rather, it is the brain, spinal cord, and spinal column along the A-P and D-V axes, in tandem with branchiomotor, sensory, cranial, and neural-crest derived neurons and neuronal signals, that these *Hox* genes act upon [22].

Hox Gene Expression in the Central Nervous System

In the *Drosophila* embryo, expression of *Hox* genes is easily examined early in development given that the embryo, or syncytial blastoderm, is polarized and determined by maternal effect genes. In vertebrates, polarization and determination of the central nervous system (CNS) is not observed because of the numerous gene duplications and lineage divergences of *Hox* genes from insects to vertebrates. However, conserved segmentation is still seen in the formation of somites, their boundaries, and specification of rhombomeres in the hindbrain and spinal cord. To test the significance, and genomic evolution, of *Hox* gene expression in these areas, loss-of-function experiments were done to test if *Hox* genes really were vital to the development of these areas in vertebrates and invertebrates. An investigation [11] on the neural regionalization of vertebrates and invertebrates exhibits that *Drosophila* genes necessary for the formation of columnar domains (i.e., epithelial tissue made of column -shaped epithelial cells) – *und, ind,* and *msh* – have orthologues in vertebrates – *Nkx (und), Gsh (ind),* and *Msx (msh)* – which are also responsible for the development of columnar domains in the central nervous system. Similarly, in the development of the hindbrain in vertebrates, a series of seven to eight rhombomeres visibly divide and are controlled by the same conserved pattern of serial segmentation observed in the *Drosophila* embryo. These patterns have been extensively studied using combinations of loss-of-function and gain-of-function analyses; these analyses revealed that there are distinct cellular and molecular characteristics that have been highly conserved from the earliest invertebrates to vertebrates. This so-called *Hox Code* is what gives each rhombomere in the premature hindbrain its identity; the *Hox Code* is vital to the specification and determination of the cells that will create the vertebrate hindbrain [9].

Hox Gene Expression in the Branchial Arches

The expression of *Hox* genes is also seen in the formation of the branchial arches, visible masses of tissue that will form the head and neck in vertebrates (subphylum Craniata). Development of the vertebrate head and neck is initiated by *Hox* gene expression in the branchial arches, which have been determined to help organize, pattern, and segment this primitive head, neck, and brain structure [23]. Hunt and coworkers [24]

termed this discovery the 'branchial *Hox* code.' The branchial *Hox* code proposes an early signaling cascade that lays the foundations of *Hox* genes, *labial* genes, and other derivatives in the formation of the neural crest. It also shows the characteristic nested expression of *Hox* genes within each developing rhombomere of the hindbrain until it reaches the r2/r3 boundary of the A-P axis. This branchial *Hox* code is also seen in the formation and restriction of *Hox-2.5* expression in the spinal cord of the mouse by 10 days post-coitum. These authors [24] concluded that the role of this nested *Hox* expression, known as colinearity, acts on the ventral regions of mouse embryos as well. Colinearity is what shows us how each gene is activated early embryogenesis. This activation is also determined by the linear order of the clusters along their specific chromosomal clusters. Overall, the significance of colinearity is due to the nesting and overlapping of *Hox* gene expression if segments that will form the branchial arches and hindbrain of the vertebrate embryo. Each segment forms because this process contributes to the formation of the A-P and D-V axis of the vertebrate embryo. The nesting and overlapping pattern of *Hox* gene expression also relates back to the Hox code whereby different combinations of Hox proteins are synthesized temporally throughout the body plan of the embryo contributing to the developing hindbrain, nascent spinal cord of the embryo, and somites (vertebral column precursors) [9, 25].

Patterning the D-V Axis in Vertebrates

Group B *Hox* genes, specifically *Hoxb3 and Hoxb9* are strongly expressed in the mouse developing spinal cord along the D-V axis forming an 'M' shape in the presumptive neural tube [9, 24]. While performing a loss-of-function experiment to test the branchial *Hox* code hypothesis, Hunt and coworkers [24] showed that loss of *Hox-1.5* activity was detrimental to normal D-V axis formation; mouse embryos showed defects in the neural crest and neural-crest derived mesenchyme but cranial ganglia developed normally. To date, scant research has been performed on the expression of *Hox* genes and their derivatives in the cranial mesoderm, which is the tissue that lies just below the neck. However, research on cranial nerves and cranial ganglia and their respective roles in the formation of the neural crest has been extensively studied in mice, chick, zebrafish, *Xenopus*, and *Drosophila* embryos [14, 19]. While there has been little research performed on the action of *Hox* genes in the cranial mesoderm - a particular kind of tissue that is derived from the paraxial mesoderm - these data, reviewed above, supports the hypothesis that *Hox* genes work to pattern the dorsal-ventral axis, the formation of the spinal cord, and the brain in vertebrates.

Hox Genes and Brain Development

In the fruit fly, *Drosophila melanogaster,* genetic mutations of the abdomen, thorax, or head results in that segment of the body taking on the identity of the other (homeotic transformation). For example, a mutation in the *Antp (Antennapedia)* gene of *Drosophila* results in an antenna being transformed into a leg. A process similar to homeotic transformation is observed in vertebrates from misexpression (e.g. duplication) of *Hox* genes in the developing axial skeleton. The ancestral murine gene, *Hoxb1,* is necessary for proper formation of rhombomere 4 (r4) in the developing hindbrain. Rhombomere 4 is necessary for proper migration of the VIIth facial branchiomotor neurons in mammals [20]. Zebrafish possess duplicate copies of *Hoxb, hoxb1a and hoxb1b*, which have similar functions as the mammalian orthologues *Hoxa1* and *Hoxb1.* Interestingly, morpholino driven down regulation of *hoxb1a* and *hoxb1b* in zebrafish resulted in striking changes to cranial/facial development due improper migration and expression of the *hoxb1* duplicates. These data demonstrate that while the *hoxb1* duplicates completely altered the organization of each rhombomere in the hindbrain itself, the orthologous copy of *Hoxb1* found in mice did not alter hindbrain segmentation when lost. Mouse *Hoxa1* null animals had phenotype similar to the loss of the *hoxb1* duplicates in zebrafish preventing branchiomotor neurons responsible for rhombomere 4 identity from migrating downstream to their specific segment; therefore these orthologues play similar roles in mouse and zebrafish neuronal development "revealing that a 'function shuffling' among paralogues has occurred during vertebrate evolution" [20].

In pufferfish [21], group two and four *Hox* genes and their duplicates are responsible for formation of r2-r5 borders in the hindbrain and for the evolutionary advantageous function called "puffing" that gives pufferfish their names. The *Hoxa2a* gene responsible for

this puffing probably resulted from duplication and divergence over time; interestingly, this gene's function in the pufferfish could be a result of evolution in the motor neurons of the pufferfish lineage given that zebrafish and other teleost fish possess a pseudogene of *Hoxa2a* that is non-functional in their genome [21]. Experimentation [9] performed on *Hoxa2* also revealed that the gene functions as a transcription factor in the formation of the cranial neural crest. The ability of *Hoxa2* to initiate the migration of neural crest cells to the second branchial arch of the hindbrain was dependent on the expression of the ancient *Ap-2* gene. If *Ap-2* is not expressed in the early development of the hindbrain, *Hoxa2* also fails to be expressed indicating that migration of the neural crest cells to the second branchial arch does not occur [9]. These observations allow for the conclusion that *Hox* genes are not only working to form boundaries and segment the hindbrain, but they are also responsible for the neural regionalization and migration of specific neural crest cells in the hindbrain and spinal cord.

Work [11, 25] on the formation of the hindbrain and spinal cord has established that hindbrain segmentation has a "striking resemblance" to the embryonic body plan of *Drosophila melanogaster.* Multiple studies on *Hox* genes in the hindbrain revealed that *Hoxb1* plays a major role in the cascade of genes that helps to form the rhombomeres in the hindbrain. Specifically, *Hoxb1* functions to form r4. In Kiecker and colleagues [25] review of chordate evolution and hindbrain segmentation, *Hoxb1* loss-of-function experiments in mice demonstrate that the identity of r4 is ectopically expressed as r2-like in the branchial arches and other segments of the hindbrain. These authors [25] also clarified that this same phenotype was present in zebrafish embryos lacking the duplicate *hoxb1a* gene, revealing evolutionary conservation and sub-functionalization as predicted by the duplication and divergence model. The reason for this identity shift is due to the inability of branchiomotor neurons (i.e. VIIth facial branchiomotor neurons) in these mutant animals to migrate to their proper anatomical positions to form the proper boundaries necessary for segmentation, neural migration, and neural regionalization of rhombomeres in the hindbrain [20, 25].

Role of Hoxb1 and Hoxa1 in the Rhombomeres of the Hindbrain

Analysis on the functions of *Hoxb1* in hindbrain development reveals that *Hoxb1* acts synergistically with *Hoxa1,* a group 1 paralog. In the vertebrate brain, a gain-of-function of *Hoxa1* leads to the transformation of r2 into a r4 identity, which is the opposite reaction of loss-of-function in *Hoxb1* demonstrating the transformation of r4 into r2-like expression. Duplicates of the ancestral *Hoxa1* and *Hoxb1* copies are found in the zebrafish embryo; when the *hoxb1* duplicate in zebrafish was analyzed in a gain-of-function experiment, the result was a transformation from r2 identity to r4 identity, which is similar to the gain-of-function experiment done on *Hoxa1* in the mammalian brain. This transformation is observed in the ancestral mammalian gene *Hoxa1*, which shows that the conservation and subsequent shuffling of functions among duplicated genes has occurred over time [15, 16, 20].

The inactivation of *Hoxa1* in the hindbrain also led to the deletion of rhombomere segments in the hindbrain. These data support the hypothesis that *Hoxa1,* coupled with *Hoxb1,* are responsible for the segmental identity of specific rhombomeres in the hindbrain. Inspection of the specific role that $Hoxa1^{null}$ mutants have in relation to *Hoxb1* in the hindbrain revealed the coupled reactions of *Hoxb1* and *Hoxa1* proposed above [15]. *Hoxa1* is one of two known paralogs expressed in the CNS. Double heterozygous and homozygous mutant embryos for *Hoxa1* and *Hoxb1* indicated that heterozygous embryos possessing one functional copy of *Hoxb1* and a mutant copy of *Hoxa1* showed dramatic alterations to r4 and a significant decrease in proper formation of r4. This shows that without *Hoxa1,* the one functional copy alone is not enough to maintain proper expression of r4 in the developing hindbrain [15]. The synergistic interactions of *Hoxb1* and *Hoxa1* can be explained by auto- and cross-regulatory pathways, which are essential for patterning the hindbrain [25].

Auto- and Cross-Regulatory Pathways Pattern the Hindbrain alongside Hox Genes

Auto-regulatory pathways are internal biological processes that are responsible for an organism's response to external stimuli whereas cross-regulatory

pathways are responsible for controlling metabolic pathways through the products made by related, but entirely different pathways. Nolte *et al.* [9] illustrated that these two pathways work in tandem with *Hox* genes to cross-regulate the expression of other *Hox* genes or sustain their own expression through auto-regulatory mechanisms. Polycomb and trithorax controlled regulatory pathways, which determine segmental expression in the fruit fly, *Drosophila melanogaster*, were also found to regulate vertebrate *Hox* gene expression in the spinal cord and hindbrain; without the mouse homologues *PcG* and *trxG* present, *Hox* gene expression is altered leading to improper expression of *Hox* genes in rhombomeric segments and skeletal defects. Hox proteins and response elements such as retinoic acid (RA), fibroblast growth factor (FGF), and Krox20 were also found to self-regulate the feedback loops that generate and maintain this segmentation [9]. The auto and cross-regulatory loops of the hindbrain and spinal cord are most evident in rhombomere 4 (r4), which as previously mentioned is patterned and segmented by *Hoxb1* and *Hoxa Hoxa1.* In addition to this, retinoic acid response elements (RAREs), fibroblast growth factors (FGFs), *Krox20, Pbx,* and Kreisler seem to aid in the signaling cascade for either auto- or cross-regulatory loops in tandem with group 1 and group 4 *Hox* genes in the hindbrain [9, 15, 26].

Retinoic Acid as a Pre-Requisite to Hox Gene Auto-Regulatory Loop

As mentioned above, paralogous group 1 *Hox* genes are primarily responsible for patterning r4 in the hindbrain. In Nolte and Krumlauf's review [9] they discuss how these patterning mechanisms showed that retinoic acid (RA), which is a derivative of vitamin A, is directly connected to embryological defects in the formation of the anterior hindbrain and spinal cord when in excess. Ectopic expression of retinoic acid also resulted in the transformation of rhombomere boundaries; whereas, the abrogation of RA in early stages of development resulted in the complete loss of the hindbrain and spinal cord. The most anterior *Hox* genes in the hindbrain are known as 3′ *Hox* genes and include *Hoxb1* and *Hoxa1*; the *Hoxb1* locus is necessary and sufficient to regulate neural and mesoderm expression, but the 3′ RARE on this locus was found to activate endogenous *Hoxb1* in the early neural ectoderm by way of an auto-regulatory loop. Investigation of this hypothesis was done by a so-called "hit-and-run" model which applies and targets a specific germline mutation in the organism; in this case, the mutation was a loss of function of 3′ RARE. Subsequently, a loss of 3′ RARE resulted in improper levels of *cis*-regulatory elements, which establish proper formation of the neuroectodermal and mesodermal expression of Hoxb1[15].

Another study done on cross-regulation and expression of *Hox* genes examined retinoic acid receptor *ß* (*Rarb)* showed that *Hoxb4* and *Hoxd4* along with the two *Hox* genes from PG1 (paralogous group 1) discussed above, required the participation of retinoic acid in order to form rhombomere boundaries and segment the primitive hindbrain and spinal cord. Data from this experiment on the mouse embryo revealed that *Hoxb4* and *Hoxd4* have congruent expression with *Rarb* in r6/r7 at around day 8.5 or 9.5 post coitum. A protein known as *Raldh2* produced results that were similar to a loss-of-function of RA [26]; loss of *Raldh2* gives rise to abnormalities along the A-P axis due to disrupted *Hox* expressions. These authors [26] also showed that there is some auto-regulatory loop function, as described above, in PG1 genes in r4 of the hindbrain, among *Rarb, Hoxb4,* and *Hoxd4* in later stages of embryonic development. Mutation of PG4 genes did not produce significant alterations, but did lead to dramatically reduced neural tube expression in the D-V axis of the mouse embryo. Therefore, RA/receptor complex binding to a distal enhancer showed that RA and PG4 *Hox* genes are responsible for D-V patterning as well as A-P patterning. Additional results from these experiments illustrate that *Hoxb4* and *Hoxd4* regulate the early expression of *Rarb* in the primitive hindbrain, neuroectodermal, and mesodermal tissues [15, 26]. While RA involvement in these processes is vital, it is not the only protein and transcription factor needed for appropriate expression in the hindbrain and spinal cord.

Fibroblast Growth Factor Signaling as a Precursor to Hox Gene Expression

Fibroblast growth factor (FGF), *Krox20,* Kreisler, and *Pbx* are all necessary for the regulation and function of *Hox* genes in the vertebrate nervous system. Gain-of-function mutations of FGF can increase

endogenous expression of *Hox* genes in the mesoderm and neural tissue. A loss of FGF results in the opposite of up-regulation in the formation of the vertebrate hindbrain and spinal cord. Specifically, research [9] performed in mouse embryos reveals that *Fgf2, Fgf3, Fgf8,* and *Fgf14* have the most effect on central nervous system development. FGF is also vital for the activation of *Hox* group genes 5-13 which are expressed in a colinear fashion as explained earlier in this review [9]. Without induction by FGF derivatives, the most caudal region of the hindbrain and the most rostral region of the spinal cord do not form properly. Thus, it appears that FGF is a prerequisite for the signaling cascade activating the expression, regulation, and segmentation action of *Hox* genes in the hindbrain and spinal cord.

Early Signaling of Krox20, Kreisler, and FGF in Hindbrain Segmentation

Fgf3, Fgf8, Krox20, and *Kreisler* are expressed in r4; expression data indicates that *Krox20* and *Kreisler* are part of an auto-regulatory loop, which activates the expression of FGF ligands and receptors. This implies that *Krox20* and *Kreisler* work to initiate proper expression of FGF ligands acting further upstream of the signaling cascade responsible for *Hox* gene expression. *Krox20* expression is also necessary to form primitive boundaries for r3 and r5; expression of this protein following formation of these rhombomeres is significantly down regulated. In order for *Krox20* to act in its specific rhombomere segments, however, it must bind to regulatory elements upstream of mammalian *Hoxa2, Hoxb2,* and *Hoxb3.* Without *Krox20* activation, no expression of these *Hox* genes occurs and formation of the rhombomere boundaries is not specified or determined. *Kreisler* functions in a very similar way but it works to form the r5 and r6 boundaries within the hindbrain. Interestingly, a loss of *Kreisler* results in inner ear and hindbrain malformations. The duplicated version of this gene in zebrafish was also found to regulate hindbrain expression; for example, if *Fgf8* expression is lost in the fish and chick hindbrain, then *Krox20* and *Kreisler* are not expressed. This leads to developmental defects in the embryo because *Hoxa2* and *Hoxb1* are not activated to form the r1/r2 boundary and the r4 boundary while *Krox20* and *Kreisler* are not able to specify and determine the r3, r5, and r6 midbrain/ hindbrain boundaries, respectively [9,27]. A loss-of-function experiment[16] on Amphioxus *hox·β. 1, hoxa2,* and *hoxa3* showed that without the action of *Kreisler* and *Krox20,* segmentally restricted expression of rhombomeres in the hindbrain does not occur properly at the r6/r7 boundary in the hindbrain. These analyses show that *Kreisler, Krox20,* and FGF are all significant in patterning the hindbrain [9, 16, 27].

Loss of Pbx Function Inhibits Rhombomere Segmentation in the Hindbrain

Pbx, a *Hox* cofactor and homeobox-containing gene encoding a nuclear protein, was also found to work alongside *Hox* genes to pattern and segment the hindbrain. A loss-of-function experiment done on *Pbx* showed that the transcription factors produced from the nuclear protein were necessary to activate segmentation of *Hox* genes in the hindbrain [2, 7]. In the zebrafish embryo, loss-of-function of *pbx2* and *pbx4* protein corresponds to improper rhombomere identity (e.g. r2 forms as r1) and formation of rhombomeres 2 through 6 is entirely lost. This is due to lack of Pbx transcriptional activation of *Hox* genes involved in segmentation in the hindbrain[28]. These experiments also pointed to the conclusion that the *Hox/Pbx* binding sites are vital to the auto- and cross-regulatory loops that regulate and initiate *Hox* genes in the hindbrain and spinal cord. As we have seen, auto- and cross-regulatory loops are what contribute to the colinear nature and nested expression of *Hox* genes and their derivatives, in the hindbrain and spinal cord while also contributing to their self-regulation and establishment in the primitive CNS of the embryo. Hox-1/Pbx binding sites are also directly involved in regulation of r4 in the zebrafish. To bring together the function of *Pbx* and the other proteins involved in the regulation of *Hox* gene expression, research[27] has also shown that loss of *pbx2* and *pbx4* in the zebrafish correlates directly and indirectly with down-regulation of FGF signaling in the hindbrain. *Hox-3* paralogs in zebrafish are also regulated by an auto-regulatory loop dependent on *Pbx* expression; therefore, in order for proper expression of *Hox* genes to occur in each rhombomere of the hindbrain, *Pbx* must be functional and normally expressed to specify and determine each rhombomere in the hindbrain. Without *Pbx,* pharyngeal arch malformations, severe anemia, and other

deformities are observed in mice and zebrafish embryos [27]. Additionally, lack of Pbx protein inhibits the expression of *Hox* genes and proper formation of primitive neuroectoderm and mesodermal tissues in the A-P and D-V axis [15, 26, 27].

Conclusion and Prospectus

Patterning the hindbrain and spinal cord is a complex and multi-signal cascade developmental event. As has been discussed, precursor signals from RA and FGF are vital to the initiation of *Hox* gene expression in the hindbrain and spinal cord. *Krox20, Kreisler,* and *Pbx* all were found to work in tandem with *Hox* genes to properly form the compartments and boundaries of the hindbrain (rhombencephalon), called rhombomeres, and the spinal cord. This review also presented information about the evolution and conservation of these *Hox* genes, and their derivatives, which come from the ancestral copies found in *Drosophila melanogaster* and were born out of the duplication and divergence model. Information presented here supports the conservation of *Hox* genes across phyla and presents the idea that *Hox* gene orthologues and homologues found in vertebrates are necessary for normal vertebrate brain development. Without *Hox* gene activity in the hindbrain (rhombencephalon) and spinal cord, the rhombomeres of the hindbrain do not form properly and the neuroepithelium surrounding the neural tube fails to close. Altered or incomplete development of the brain could result in spina bifida, anencephaly, exencephaly, or craniorachischisis depending on which stage of neurulation the malformations in the developing brain occur [2, 28]. To better understand the role of *Hox* genes in all parts of the brain, more research needs to be conducted on the formation of the cranial mesoderm, the tissue that sits just below the neck, and how it participates in the formation of the spinal cord, the neural tube, and some portions of the hindbrain.

The vertebrate orthologue *Msx* (muscle segment -related homeobox) was found to be expressed in bones, teeth, neural crest cells, and the placodes of the brain. *Msx* also plays a necessary role in the formation of neuronal circuitry of the brain along with FGF, *Krox20*, and BMP. Future research on these orthologues could uncover potential roles of *Msx* in the formation of the vertebrate skeleton and, subsequently, could find that *Hox* genes also play a role in limb and axial skeletal formation. The presence of *Msx*, FGF, *Krox20*, and BMP in the neuronal circuitry of the brain allows for questions about whether *Hox* genes help form other parts of the vertebrate brain during development and throughout the life of the organism. Understanding the roles of *Hox* genes and conserved derivatives discussed in this review could open the door to research on treatment for neuronal and mental disorders. For example, RA is a vital precursor that initiates the signaling cascade for *Hox* gene segmentation in the brain. If a deficiency in RA or an excess amount of RA is present, could we induce or repress RA in the brain medicinally to prevent malformations in the brain? Further research on *Hox* gene products, and their homologs, as pharmaceutical targets may help guide us in treating many neural disorders that are a result of malformations and improper segmentation.

Acknowledgements

This work was supported by the Department of Science and Mathematics of Judson University (S. G. S. and J.O.H) and by funds from the William W. Brady Chair of Science endowment (J.O.H).

Competing Interests

The authors declare no competing interests.

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