Riding the crest to get a head: neural crest evolution in vertebrates

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Abstract:

In their seminal paper, Gans and Northcutt (1983) proposed that evolution of the vertebrate “New Head” was enabled by advent of the neural crest and cranial placodes[1]. The neural crest is a stem cell population that arises adjacent to the forming central nervous system and contributes to important cell types including components of the peripheral nervous system, craniofacial skeleton, and elements of the cardiovascular system[2]. In the past few years, the New Head hypothesis has been challenged by the discovery in invertebrate chordates of cells with some but not all characteristics of vertebrate neural crest cells[3-7]. Here, we discuss recent findings regarding how neural crest cells may have evolved during the course of deuterostome evolution. The results suggest that there was progressive addition of cell types into the repertoire of neural crest derivatives throughout vertebrate evolution[8]. Novel genomic tools have enabled higher resolution insight into neural crest evolution from both a cellular and gene regulatory perspective[9,10]. Together, these data provide clues regarding the ancestral neural crest state and how the

neural crest continues to evolve to contribute to the success of vertebrates as efficient predators.

Introduction

Nearly 40 years ago, the New Head hypothesis proposed that the complexity and elaboration of the vertebrate head was a consequence of the advent of the neural crest and cranial placodes (Figure 1)[1]. These new cell types enabled assembly of the craniofacial skeleton and a novel sensory system, which in turn allowed expansion of the anterior neuroepithelium into the vertebrate brain (Figure 1) [1,7,11,12]. The morphological characters that arise from the neural crest and cranial placodes also allowed for the transition from a predominantly filter feeding lifestyle of invertebrate chordates to active predation of vertebrates. The multipotent neural crest is a synapomorphy, shared and derived in all vertebrates, that is intimately linked to the evolution and diversification of vertebrates.

Neural crest cells are characterized by their multipotency, migratory abilities, and differentiative capacity[2]. Early in development, the neural crest arises in the dorsal most aspect of the forming central nervous system, from which it undergoes an epithelial to mesenchymal transition (EMT) to delaminate from the neuroepithelium. These cells then migrate extensively throughout the early embryo to give rise to diverse derivatives depending on their final location (Figure 2a). Four main subpopulations of neural crest cells exist along the anteroposterior axis of jawed vertebrates (Box 1): cranial, vagal, trunk, and lumbosacral (Figure 2b,c)[13].

The cranial neural crest arises from the anterior central nervous system ~~levels~~ -- forebrain, midbrain, and hindbrain. Whereas anterior-most cranial neural crest form the frontonasal skeleton, more posterior cranial crest cells populate the pharyngeal arches to form bone and cartilage of the jaw, middle ear, and neck (Figure 1)[14-16]. The vagal neural crest predominately contributes to the enteric nervous system and portions of the heart including the cardiac outflow tract, heart valves, cardiac ganglia, and cardiomyocytes[17-20]. The trunk neural crest gives rise to neurons and glia of the dorsal root and sympathetic ganglia[21]. Finally, the lumbosacral neural crest gives rise to portions of the enteric and sympathetic nervous systems (Figure 2b,c)[22].

Underlying the development of these subpopulations is a pan-neural crest gene regulatory network (GRN) that describes the regulatory interactions at each stage of neural crest development (Figure 2d)[2,10,23,24]. Superimposed on this global GRN are axial specific subcircuits that are "plugged in" to core circuitry to imbue region-specific developmental potentials[25]. Comparative studies across diverse vertebrates provide an approach for probing how these axial level specific subcircuits may have evolved[8]. Identifying changes in gene regulatory programs across vertebrate evolution can inform upon evolutionary change and morphological novelties found that may have led to emergence of novel structures including the vertebrate New Head[26].

In addition to the vast number of derivatives the neural crest will generate, they will also give rise to a multipotent population of cells that cover peripheral nerves throughout the body called Schwann cell precursors (SCPs)[27]. SCPs have been reported to detach from peripheral nerves and become Schwann cells, autonomic neurons, neuroendocrine cells, melanocytes, and other neural crest-derived cell

types[28-31]. SCPs not only have important implications in regenerative medicine but also provide insights into the evolutionary origin of the neural crest[32-34].

While invertebrate chordates lack *bona fide* neural crest cells, they do possess cell types that have either aspects of the cellular morphology or shared gene regulatory programs with the neural crest[3,4,6,35]. However, these cell types also lack key features of neural crest cells like multipotency, extensive migratory capacity, and the ability to give rise to ectomesenchymal structural elements. That said, evidence from invertebrates helps to elucidate the ancestral state of the neural plate border, the neural crest, and the neural crest gene regulatory network (Figure 2d)[26,36].

In this review, we examine the substantial contribution of the neural crest to the evolution of novel vertebrate traits. We discuss the presence of neural crest-like cell types in invertebrates, implications of the organization of the lateral part of the neural plate, and how addition of novel gene regulatory programs may have influenced advent and further specialization of the vertebrate neural crest. We speculate that the neural crest may have evolved in a stepwise fashion by progressive refinement of GRN subcircuits along the anterior-posterior axis during vertebrate evolution. Finally, we discuss the increasing complexity of neural crest derivatives and how co-option of gene regulatory programs throughout the course of vertebrate evolution has continued to imbue the vertebrate body plan with novel features. Given ongoing findings regarding the gene regulatory programs underlying multipotency, migration, and differentiation of neural crest cell and cranial placode cell types, the New Head hypothesis continues to develop and evolve.

The neural crest gene regulatory network

Development of animal body plans is encoded in the regulatory genome, and modifications in gene regulatory programs lead to evolutionary change[37,38]. Changes in morphological characters are driven by alterations in gene regulatory modules by gene innovation, gene duplication, and/or co-option of regulatory information from different tissues. Gene regulatory networks describe the regulatory interactions formed by transcription factors and cis-regulatory elements at each stage of development in a particular cell type[39]. Integrated in these regulatory networks are cellular interactions mediated by signaling and receptor molecules and cues that dictate gene expression and function. Gene regulatory networks are broken into modules of regulatory information that include the genes and interactions that function at different time points of development. The given regulatory interactions in a cell at any given time represent its regulatory state[40]. Conservation and changes in gene regulatory logic are at the core of our understanding of what drives evolution of the neural crest[2,9,26,41].

Formation of neural crest cells is controlled by a feed-forward gene regulatory network that controls their induction, migration, and differentiation (Figure 2d). As development proceeds, the neural crest progresses through successive regulatory states from specification to EMT to migration and ultimately to differentiation (Figure 2a)[24]. Underlying each of these developmental milestones is a core gene regulatory network that describes the key interactions at each stage of development. The GRN is composed of developmental modules comprised of transcription factors and signaling molecules that interact in order to drive discrete steps of development (Figure 2d). By unraveling these networks, we can begin to understand the logic dictating how the neural crest arises, differentiates, and may

have evolved to form unique derivatives in jawed vertebrates[2,26]. The current view of the neural crest gene network has emerged over time and encompasses numerous organisms from basal vertebrates to mice and human pluripotent stem cells (hPSCs)[2,23,24,42,43].

Initiation of neural crest formation begins during gastrulation, as a series of signaling events including Wnts, FGFs, and BMPs refine the border between the forming neural and non-neural ectoderm[44-48]. These signaling interactions promote regionalization along the mediolateral axis of the developing embryo and, at the presumptive neural plate border, activate downstream specification gene regulatory modules that include transcription factors such as *Zic1, Msx1, Tfap2,* and *Pax3/7* (Figure 2d) [44,46,48-55]. Once the neural plate border has formed, the neural crest becomes specified as exemplified by expression of transcription factors including *SoxE, FoxD3, Snai1/2,* and *Tfap2* [52,54,56]. Following specification, neural crest cells undergo an epithelial-to-mesenchymal transition to delaminate from the forming central nervous system. This delamination process is tightly controlled by regulatory interactions that coordinate a "Cadherin switch" to allow for the de-adhesion of precursors from the neural tube[57-62]. Once free from the central nervous system, the neural crest cells activate a migratory gene network module to migrate extensively throughout the embryo[10,63-65]. Upon reaching appropriate locations, they activate differentiation gene batteries that mediate differentiation into distinct derivatives based on their anteroposterior location (Figure 2d) [2].

Along the anteroposterior body axis, the neural crest can be subdivided into four main subpopulations: cranial, vagal, trunk, and lumbosacral (Figure 2b)(Box 1). While neural crest cells at all axial levels form some common cell types like melanocytes and glia, there also are neural crest-derived structures that are unique

to particular axial levels (Figure 2c). These unique derivatives are the consequence of deployment of axial-specific circuits that drive distinct fates related to their anteroposterior site of origin[13,66]. For instance, in amniotes, only the cranial axial level is able to give rise to skeletogenic fates *in vivo*[15,16]. Recently, a unique cranial specific circuit was found in chicken embryos that includes transcription factors *Brn3c, Lhx5, Dmbx1, Tfap2b, Sox8,* and *Ets1*[25]. When components of this circuit (*Sox8, Ets1,* and *Tfap2b)* were ~~placed~~ ectopically expressed in the trunk neural crest, these were sufficient to impart skeletogenic potential by enabling trunk neural crest cells to form cartilage nodules after grafting to the head[25]. However, since this circuit is insufficient to drive these fates when the trunk neural crest remained in the trunk, cranial-specific environmental cues and yet-to-be defined signals likely participate in the axial diversification of the neural crest. Still, u~~U~~nderstanding how subcircuits like this arose and evolved is important for elucidating how the neural crest was able to give rise to morphological novelties in different parts of the body.

At the vagal axial level, neural crest cells contribute to the heart and gut, forming the outflow tract septum and enteric nervous system (Figure 2c). When the anterior vagal (called “cardiac”) neural crest is ablated in chick embryos, the outflow tract septum which connects the heart to the lungs fails to form properly, resulting in mixing of oxygenated and non-oxygenated blood, a defect highly reminiscent of a common human congenital heart defect[67]. Only cardiac neural crest cells have the ability to form the outflow tract septum whereas trunk and cranial neural crest cells cannot do so. Recently, a neural crest gene subcircuit, comprised of transcription factors *Tgif1, Sox8,* and *Ets1*, was shown to be specific to this axial level and, when introduced ectopically, was able to confer this

developmental potential to trunk neural crest cells grafted to the cardiac crest region[68]. Thus, similar to the cranial crest-specific subcircuit that can confer cartilage forming ability, the cardiac crest-specific subcircuit appears to confer the ability to form a different derivative, mesenchymal cells of the outflow tract septum, onto a neural crest subpopulation in a region-specific manner. Future studies focusing on axial specific subcircuits in other neural crest subpopulations hold the promise of clarifying how these subcircuits act to drive cell type diversification along the anteroposterior body axis.

Other likely important players in the formation of distinct subpopulations along the anteroposterior axis are the *Hox* genes (Box 2). Differential *Hox* gene expression and their interactions with other neural crest gene network genes may be sufficient to account for subtle gene network differences observed along the anteroposterior axis and may act to modulate neural crest axial level differences in cell fates[69].

At its core, the neural crest gene regulatory network is remarkably similar in overall architecture and composition across all vertebrates, though species specific differences enable flexibility in morphological traits such as craniofacial features. Still, the overall network is vastly similar and adaptable such that modular components, such as axial specific subcircuits and differentiation gene batteries can be “plugged in” to the network to add to its evolvability and adaptability.

Origins of the neural crest GRN

Across vertebrates, groups of transcription factors, including *Pax3/7, Msx1, Zic1, Tfap2, Snai1/2, FoxD3*, and *SoxE*, and their regulatory interactions, are conserved in terms of expression in the neural crest and placement in a pan-vertebrate gene

regulatory network (Figure 2d) [2,26,70]. These factors serve as a kernel that functions to establish the neural plate border and promote neural crest multipotency and migration. However, important gene regulatory differences between jawed (gnathostome) and jawless (cyclostome) vertebrates provide clues as to how novel cell types may have evolved under the umbrella of the neural crest. Both lamprey and hagfish are cyclostomes and form a monophyletic sister group to the jawed vertebrates[71-73]. ~~These jawless vertebrates are reminiscent of a "living fossil", as their body plans have remained relatively unchanged for over 500 million years. But~~ Ff~~f~~ar more is known about the gene regulatory network of lamprey than hagfish neural crest since it is very difficult to obtain live embryos from the latter[74-77].

Interrogation of neural crest gene network conservation and changes in the sea lamprey can provide insight into ~~the ancestral neural crest gene network~~the formation of morphological novelties. For instance, work in the sea lamprey has shown that transcription factors *Ets1* and *Twist*, two major players in the pre-migratory/migratory regulatory module of the neural crest, are absent from the early neural crest GRN[70]. *Ets1*, which is essential for neural crest specification in jawed vertebrates, is instead expressed in late neural crest derivatives within the branchial arches as well as dorsal root ganglia, a trunk-derived neural crest cell type[8,70]. This represents a significant change in the gnathostome gene network from the early ancestral vertebrate gene regulatory network where *Ets1* was co-opted from later in development, or a more distal part of the network hierarchy, to drive specification of gnathostome neural crest specification potentially leading to novel cell fates. Another transcription factor, *Twist*, which is essential in frogs but dispensable in mice for neural crest development, is absent in lamprey migratory neural crest but present in later derivatives, providing another example of a distal

225 node of the network that was co-opted to more proximal parts of the neural crest
226 gene network in gnathostomes[70].

227 A comparative analysis of the neural crest GRN that governs the ability of cranial
228 neural crest cells to form the facial skeleton between amniotes and other
229 vertebrates has shown that neural crest gene network components have been
230 progressively added to the neural crest during the course of vertebrate evolution[8].
231 Several of the genes that are cranial crest specific in amniotes, instead of being
232 added *de novo* to the cranial neural crest, appear to have been co-opted from more
233 distal parts of the gene network to proximal modules at all axial levels then
234 progressively restricted to the cranial axial level during the course of gnathostome
235 vertebrate evolution[8]. Thus, basal vertebrates appear to have had a “basic” neural
236 crest gene network that was relatively uniform along the body axis. With
237 progressive evolution, genes were added and subcircuits built at different axial
238 levels, resulting in subpopulations of neural crest cells with different migratory
239 pathways and the ability to form distinct derivatives. The results of these recent
240 comparative studies of gene regulatory control of neural crest development suggest
241 that the New Head, as opposed to arising at the base of vertebrates *in toto*, arose
242 progressively during the course of vertebrate evolution.

243 Two recent stories shed light on vertebrate-specific gene innovations and gene
244 duplication events that enabled expansions and diversification of the neural crest.
245 In one recent study, Scerbo and Monsoro-Burq (2020) show that the loss of
246 vertebrate-specific Ventx2, an ortholog of mouse Nanog, leads to a loss of the
247 neural crest multipotent state and skeletogenic potential[78]. This genetic
248 perturbation results in a neural crest that can only give rise to sensory neurons and
249 pigmentation, similar to neural plate border derivatives found in invertebrate

chordates. Further, Scerbo and Monsoro-Burq show that mouse Nanog is able to rescue the craniofacial phenotype of the Ventx2 depletion demonstrating a functional equivalence of Ventx2 and Nanog. Another recent study reports on the significance of genome duplication events that led to the expansion and diversification of neural crest subpopulations during vertebrate evolution. Endothelin ligands and receptors are unique to vertebrates and two rounds of genome-wide duplication events that occurred in basal vertebrates, the *Edn* signaling pathways components diverged and became specialized in order to expand the neural crest repertoire[79,80]. These examples provide further evidence that throughout chordate evolution, the neural crest gene regulatory network was progressively elaborated to give rise to vertebrate novelties.

The advent of new systems-level techniques that are amenable to many research organisms has shed light on regulatory network changes and additions that drove the evolution of novel morphological characters of the neural crest. Initially, neural crest gene network interactions were studied by taking a candidate approach to identify genes expressed in the neural crest and then testing the effects of gene knock-down on other known neural crest markers. Recently, next-generation sequencing techniques including bulk and single cell RNA-seq, ChIP-seq, CUT&RUN, and assays for transposase-accessible chromatin using sequencing (ATAC-seq) have been applied to the study of neural crest development in several species ranging from jawed to jawless vertebrates[9,10,81,82]. By comparing the global gene regulatory networks between these diverse vertebrates, it is possible to glean changes in the neural crest that have occurred as a function of evolutionary time.

Lending to our understanding of how novel programs evolve, recent single cell analyses throughout neural crest development in the mouse suggests a three-step

fate selection mechanism where multipotent neural crest cells co-activate opposing regulatory programs for different fates, followed by repulsion of one program and commitment to a distinct fate[82]. From an evolutionary perspective, it is interesting to speculate that mechanisms such as these are at play to give rise to novel derivatives by co-option of fates from other cell lineages.

The gene regulatory network is not only useful for assessing regulatory changes that have occurred in the vertebrate lineage, but also for uncovering the homology of similar cell types in invertebrate chordates that provide clues regarding the origins of the neural crest (Figure 2d). Recent work in invertebrate chordates based on comparative gene regulatory network analyses suggest a more primitive origin of the neural crest than previously assumed.

Comparative approaches at gene network dissection help to uncover the foundations for evolutionary change via changes in linkages or subcircuits within the gene network, which in turn can inform upon new morphological novelties. Using information gathered from comparative regulatory analyses of GRNs, one can infer whether different morphological characters may have convergently evolved by parallel deployment of differentiation gene batteries. The more we learn about the neural crest gene network, the more we understand how mechanistic changes in the regulatory program have influenced the evolving vertebrate body plan and New Head.

Invertebrate neural crest-like cells

Central to the understanding of vertebrate evolution is uncovering when *bona fide* neural crest first appeared. Recent evidence from invertebrates suggests that the neural crest evolved in a step-wise fashion throughout the evolution of

deuterostomes. Cell types that share neural crest features such as molecular signatures, location of origin, and derivatives may represent a lineage that is homologous to the neural crest, co-opted from other tissues and incorporated into an evolving neural plate border population[83]. While one cannot rule out that these cell types arose by means of convergent evolution, evidence suggests that the cell types and regulatory programs implemented in the formation of these cell types in invertebrate chordates may represent an ancestral state. As a case in point, the neural crest-like cell types that have been identified to date lack multipotency and extensive, long-range migratory ability. Comparative gene regulatory studies have now enabled the investigation into neural crest-like cell type evolution in invertebrates by assessing the presence of regulatory programs in cell types that don't necessarily have all distinguishing characteristics of the vertebrate neural crest cells like multipotency or long-range migratory ability yet share some common gene signatures. Future comparative gene regulatory studies aimed at uncovering why invertebrate chordate neural crest-like cells lack multipotency programs is important for understanding the origins of vertebrate neural crest.

In urochordates, cell types similar to pigment cells and neurons have been found with gene regulatory similarities to neural crest and cranial placode cells (Box3) that reflect a preliminary neural crest gene network (Figure 2d). This network consists of homologues to *Id, Zic, Pax3/7, Mitf, Msx, Snai, Ets1,* and *FoxD* that are expressed in cells that are located at edges of the lateral neural plate; however, the cells within these expression domains neither migrate extensively nor retain multipotency properties to give rise to a wide variety of derivatives (Figure 2d)_[4,35]. ~~These cell types include migratory cells, derived from the A7.6 lineage, that escape the developing neural tube in the ascidian *Ecteinascidia turbinata* to make pigment~~

~~cells.~~ Further, progenitors of the pigmented cells in the lineage of otolith and ocellus~~Another population of pigmented sensory cells of the otolith and ocellus~~, derived from the a9.49 cell lineage in the ascidian *Ciona intestinalis,* normally remain in the central nervous system but have the ability to extensively migrate upon misexpression of *Twist*[4]. Finally, a ~~neuron~~ cell type that ~~cell type, bipolar tail neurons (BTNs), that~~ originates from the b8.20 and b8.18 cell lineages, has been found to arise in the posterior lateral plate border, then migrate away from the central nervous system to give rise to neurons call bipolar tail neurons (BTNs) that are similar to vertebrate sensory neurons of dorsal root ganglia. BTNs express transcription factors *Neurog* and *Islet,* which are both required for vertebrate sensory neuron differentiation, suggesting that these cells represent neural crest-like cells or placode-like cells on both a morphological and molecular level[3].

Beyond derivatives formed from neural crest-like cells, it was also recently shown that there are parallels between the compartmentalization of the lateral plate in *Ciona* and the neural plate in vertebrates. Both systems require similar network interactions in order to drive the formation of different sub-domains across the lateral organization of the neural plate, including *Six1/2, Pax3/7*, and *Msxb*[84]. In urochordates, this organized lateral plate will give rise to sensory cells that are similar to both vertebrate cranial placode and neural crest derivatives. Furthermore, relatively minor gene network perturbations lead to a fate switch of one sensory cell type to another. These data suggest that cranial placodes and neural crest may have arisen from a common precursor population and only after reorganization of the lateral plate did the pre-placodal domain become distinct from the rest of the neural plate border (Box 3) [84].

In the cephalochordate amphioxus, homologues of neural plate border specifiers *Msx, Zic,* and *Pax3/7* are expressed in cells that are found in the lateral edges of the neural plate[85]. Amphioxus also exhibits expression of *Snail* in an ependymal cell in the neural tube, but this cell type is not migratory[86]. However, while many of the genes that are expressed in the neural crest in vertebrates are present in the cephalochordate genome, none of the cells that express these genes are multipotent, migrate, or differentiate into neural crest cell types[87,88]. Still, it may be possible that the amphioxus possesses cells with some homologyue to neural crest cells, such as pigment cells of the ocellus, but this still requires further investigation.

The New Head hypothesis states that neural crest and cranial placodes arose in rudimentary form in urochordates, but new evidence suggests a more primitive origin for neural crest-like cells in sea urchins, a basal deuterostome. Recently, it was reported that a population of neurons in the sea urchin, *Lytechinus variegatus*, share homologous similar features to BTN cells of urochordates, which share features with neural crest cells[89]. These ciliary band neurons arise at the border of the neuroectoderm and non-neural ectoderm in the sea urchin larva, migrate from bilateral sites of origin, express *ngn*, and differentiate into afferent sensory neurons that are required for swimming behavior[89]. One possible interpretation is that appearance of the neural crest lineage was not so sudden but rather, a neural crest-like condition was a continuous character that existed in multiple states and was remodeled in a step-wise fashion over the course of deuterostome evolution. A caveat, however, is that one cannot rule convergent evolution of some of these cell types.

From the data in invertebrates thus far, we can elaborate on crucial points in the New Head hypothesis involving origin of the neural crest and cranial placodes (Figure 3). One could speculate that regulatory programs were progressively co-opted from neighboring tissues by means of germ layer rearrangement and compartmentalization of the neural plate. The vertebrate acquisition of a multipotent state and more complex gene regulatory network modules resulted in a neural crest that gives rise to more elaborate derivatives, including ectomesenchymal cell types, by co-option of new differentiation gene batteries. It is also important to note that homologies drawn from molecular similarities alone are not conclusive but will need to be supplemented with more information on morphological and behavioral similarities, as well as more expression and genome-wide similarities.

Neural crest cell types in vertebrates

Comparisons between two major groups of living vertebrates, the jawed (gnathostome) and the jawless (cyclostome) vertebrates, have shed light on the origin of the vertebrate neural crest and the means by which it has evolved. By comparing extant vertebrate organisms, it can be concluded that emergence of the vertebrate lineage was accompanied by the introduction of neural crest cells that acquired novel derivatives, multipotency, and extensive migratory ability.

By studying the neural crest in these two groups, shared, derived traits of the early neural crest can be identified. Neural crest cell types that are shared among vertebrates include neurons and glia of the peripheral nervous system, pigment cells, cellular pharyngeal cartilage, cardiac valves, and chromaffin cells. However, many of these cell types, including cranial derivatives such as jaws and

odontoblasts that produce dentin, a vagal-derived enteric nervous system, and trunk derived sympathetic ganglia, are absent in cyclostomes[34,90]. These most likely arose in stem gnathostomes by modifications in gene regulatory network architecture that gave rise to new morphological novelties. Emergence of these novel gnathostome cell types also coincides with the refinement of neural crest axial subpopulations and their unique developmental potentials (Box1).

Assembly of axial specific transcriptional circuits occurred progressively throughout vertebrate evolution to give rise to distinct axial derivatives. Skeletogenic potential is a derived feature, arising later in vertebrate evolution but initially emerging along the entire anteroposterior axis[8]. Addition of cranial-specific circuits resulted in progressive restriction of skeletogenic fate to the cranial population in amniotes. In support of this, invertebrate chordate neural crest-like cell types lack skeletogenic potential but possess the ability to form pigment cells and neurons, traits common to all neural crest axial levels[3,4]. These data suggest that the New Head arose progressively by first acquiring skeletogenic potential at all axial levels then becoming restricted to the cranial levels by addition of neural crest gene network nodes.

While jaws are a clear gnathostome novelty, the origin of the vertebrate head skeleton did not depend on the evolution of a new skeletal tissue, but rather on the spread of this tissue throughout the head and modification of the anterior pharyngeal arches[91]. While ectomesenchymal derivatives and gnathostome novelties such as jaws have been restricted to the cranial neural crest, odontoblasts, or cells that produce dentin, may have originated along the length of the body axis before becoming restricted to cranial regions. While the trunk neural crest is often regarded as non-skeletogenic in gnathostomes, it has recently been

shown to give rise to an ectomesenchymal cell type in the little skate, *Leucoraja erinacea*[92]. Using DiI cell lineage tracing, Gillis and colleagues revealed that the dermal denticles in the trunk region are derived from neural crest cells, thus revealing a trunk origin for odontoblasts. A small circuit of transcription factors that is sufficient to confer ectomesenchymal ability in the trunk of amniotes was recently shown to be expressed along the length of the little skate anteroposterior axis, lending support to the presence of ectomesenchymal potential in the skate trunk neural crest cells[8].

The enteric nervous system in gnathostomes arises from vagal and sacral neural crest populations[17,28]. Recent evidence from Green and colleagues (2017) shows that lamprey lacks a vagal subpopulation of neural crest and only possesses cranial and trunk neural crest populations; however, the sea lamprey has an enteric nervous system. To determine the evolutionary origin of the vertebrate enteric nervous system, they performed DiI lineage tracing and found that the enteric neurons of the lamprey are derived from late migrating trunk neural crest-derived Schwann cell precursors[34]. Further gene regulatory analyses of neural crest subpopulations across diverse species will augment understanding of the evolution of the neural crest and the vertebrate body plan.

Conclusions

Vertebrates, which emerged during the Cambrian explosion more than 500 million years ago, are the most species-rich and geographically dispersed deuterostomes in the world today. This can largely be attributed to the elaboration of their head skeleton and sensory system which facilitated expansion of the brain and active, efficient predation. This vertebrate New Head was enabled by the

advent of multipotent neural crest and cranial placode cells (Figure 1). Comparisons between the embryonic development and gene regulatory networks of the two main groups of living vertebrates, jawed vertebrates and their sister group, the jawless vertebrates yield insights into the state of the neural crest in the last common vertebrate ancestor.

With advances in evolutionary and developmental biology and the ability to investigate questions in emerging research organisms, we can begin to dissect the New Head at a deeper level. Furthermore, systems-level approaches enable unraveling of gene regulatory networks and their evolutionary implications on morphological novelties and the ancestral vertebrate state.

Questions remain of how neural crest cells integrated into the invertebrate body plan to form the “new head”. Interactions between the emerging neural crest, mesoderm, and the developing CNS were crucial to the elaboration of the craniofacial novelties found in vertebrates. Yet, what factors are responsible for this integration has yet to be uncovered. Recent evidence supporting the New Head hypothesis infers that a rudimentary neural crest and cranial placodes arose from a common population of cells lateral to the neural plate. With continued regulatory modifications, germ layer rearrangements, and acquisition of the neural crest specification gene regulatory network kernel, the neural crest evolved into a multipotent and migratory population in stem vertebrates. Further interrogation of the role of peripheral nerves in dictating and guiding proto-neural crest cells to novel destinations, including craniofacial features, as is seen in the development of the lamprey enteric nervous system from Schwann cell precursors could be of interest in understanding the incorporation of new cell types in the invertebrate body plan.

Ancestral vertebrates possessed a neural crest that was multipotent, more homogenous in molecular makeup along the anteroposterior axis, and capable of producing ectomesenchymal cell fates. With continuing evolution and increasing complexity, co-option of gene network circuits, gene duplications, and neofunctionalization led to further elaboration of the core neural crest gene regulatory network to give rise to a vast array of neural crest cell types resulting in the vertebrate New Head and other gnathostome-specific structures such as an outflow tract septum and vagal neural crest-derived enteric nervous system. As new cell types appear to be added to the neural crest with continuing evolution, we speculate that the neural crest will continue to elaborate and improve vertebrate features to make an ever better head, heart, and gut.

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Competing interests:

The authors declare no competing interests.

Figure legends:

Figure 1. Core elements of the New Head Hypothesis. New Head hypothesis proposed that the complexity and elaboration of the vertebrate head was a

consequence of the advent of the migratory cranial neural crest and cranial placodes. These new cell types enabled assembly of the craniofacial skeleton and a novel sensory system, which in turn allowed expansion of the anterior neuroepithelium into the vertebrate brain. The morphological characters that arise from the neural crest and cranial placodes also allowed for the transition from a predominantly filter feeding lifestyle of invertebrate chordates to active predation of vertebrates. During development, the cranial neural crest will emigrate from the neural tube to populate the forming head (a). Distinct neural crest migratory pathways are color coded to match the craniofacial skeleton derivatives they will form in the adult (b) (adapted from Couly et al, 1998 and Santagati and Rijli, 2003)[14,93]. Formation of the cranial placodes (c) is also a defining feature of the vertebrate New Head (adapted from Depew and Olsson, 2008) [94].

Figure 2. Neural crest development and gene regulatory networks. (a.) Developmental milestones of neural crest formation include formation of the neural plate border, specification of the neural crest, delamination from the central nervous system, and migration to often distant locations to give rise to diverse cell types (adapted from Martik and Bronner, 2017) [2]. (b.) Along the anteriorposterior body axis, the neural crest is broken into four main subpopulations: the cranial, vagal, trunk, and sacral. Dotted line (a) represents location of the section depicted in panel a. (c.) Depending on their final axial location, the neural crest will differentiate into unique derivatives. (d.) Underlying the development of the neural crest is a pan-neural crest gene regulatory network that is composed of hierarchically organized modules of signaling molecules and transcription factors that dictate each process. Regulatory information gleaned from neural crest-like

cells in tunicates have now enabled the investigation into neural crest-like cell type evolution (adapted from Green, et al, 2015) [26].

Figure 3. Cladogram of extant deteurostome neural crest-related characters and evolution. Presented is a model for the evolution of neural crest features throughout deuterostome evolution. Labels to the right indicate monophyletic groupings. Highlighted character changes within a stem group are listed by bullet points. Animal illustrations adapted from Martik, et al 2019[8] or Biorender.com.

Boxes:

1. Axial regionalization of the neural crest

Neural crest cells arise within the forming neural tube from the level of the posterior diencephalon ~~along the length of the body axis~~to the lumbosacral region of the developing embryo. However, there are regional differences in migratory pathways and cell types into which they differentiate depending on their axial level of origin. Based largely on interspecific grafting experiments performed in bird embryos ~~(rev Le Douarin, 1982),~~ the neural crest can be subdivided into populations termed cranial, vagal, trunk and lumbosacral[95]. Cranial neural crest arises at the level of the forebrain to hindbrain adjacent to the forming ear; these cells form much of the craniofacial skeleton and also contribute to glia and some neurons of cranial ganglia. More caudally, vagal neural crest cells arise from mid-otic to somite 7 levels of the neural tube; these cells migrate to the heart, forming the aorticopulmonary and interventricular septa and cardiomyocytes, and to the gut to form the enteric nervous system (ENS). Trunk neural crest cells arise adjacent to somites 8-28 and form sympathetic and dorsal root ganglia. Lumbosacral neural

crest cells arise in the tailbud region; like vagal cells, they migrate to the gut, contributing to the most caudal portions of ENS. All subpopulations generate melanocytes of the skin. While neural crest regionalization is largely conserved across gnathostomes, there are differences in the precise position of “borders” between adjacent subpopulations depending upon species [96,97].

Neural crest subpopulations differ in their developmental potential as shown by grafting to ectopic sites. For example, avian trunk neural folds transplanted into cranial regions appear to lack the ability to form craniofacial cartilage. In the reciprocal experiment, cranial crest grafted to the trunk formed some normal trunk derivatives like sensory and sympathetic ganglia but also differentiate into ectopic cartilage nodules. Similarly, vagal neural crest cells grafted to the trunk form normal trunk derivatives but also invade the gut to form enteric ganglia, something trunk neural crest cannot do. Thus, there appear to be intrinsic differences in the ability of neural crest cells from different axial levels both in terms of their migratory response to the environment and ability to differentiate into certain cell types ~~(rev Le Douarin, 1982)~~ [95].

2. Hox regulation of neural crest patterning

Hox genes are expressed in the developing central nervous system (CNS), beginning in the hindbrain and continuing down the spinal cord, in a rostrocaudal order that mirrors their order along the chromosome. As neural crest cells migrate away from the hindbrain, they express the same Hox gene code as the neural tube site of origin, which is then observed in the peripheral nervous system and branchial arches into which they migrate. This led to the idea that Hox gene identity of the neural crest may be pre-patterned, such that they “carry” positional information

acquired in the hindbrain to the periphery. This would also suggest an important role for the Hox gene code in the formation of distinct axial subpopulations of the neural crest. Hunt and colleagues tested this possibility by ablating the hindbrain neural crest and found that the branchial arches still maintained autonomous Hox gene expression in the absence of the neural crest[98] ~~(Hunt et al., 1995)~~. Moreover, neural crest cells that migrated from a Hox-expressing region of the hindbrain were found to turn off their Hox expression if migrating into a Hox-negative region, thus exhibiting plasticity in Hox gene expression depending upon their environmental context [99]. Interestingly, FGF8 signaling from the midbrain/hindbrain (isthmus) region controls Hoxa2 expression, which in turn acts as a selector gene governing formation of second branchial arch structures[100].

Absence of Hox gene expression in the midbrain is also critical for proper facial formation. Creuzet, LeDouarin and colleagues (2005) showed that the Hox-negative anterior neural crest which gives rise to first branchial arch structures like the jaws plays a critical role in formation of the facial skeleton and brain. Forced expression of Hox genes (*Hoxa2*, *Hoxa3,* and *Hoxb4*) in anterior neural fold inhibits facial skeleton development as does ablation of the anterior neural folds, which reduces FGF8. Furthermore, Hox-positive neural folds cannot replace ablated Hox-negative neural folds. Anterior neural fold ablation reduces Fgf8 expression in the ventral forebrain and ectoderm of the first branchial arch[101]. These experiments emphasize the importance of signaling centers in controlling gene expression and the necessity of keeping off the caudalizing influence of Hox gene expression to maintain anterior cranial identity.

3. Cranial placode evolution

Cranial ectodermal placodes arise in the head ectoderm as thickenings in the future epidermis of early vertebrate embryos[102]. These placode cells then become internalized by ingression or invagination and differentiate to form sensory structures like the inner ear, nose, and lens as well as the neurons of cranial sensory ganglia (Figure 1). Like neural crest cells, ectodermal placodes are one of the defining features of vertebrates, raising questions about how they may have evolved. Only vertebrates, including basal jawless vertebrates, have ectodermal placodes. However, non-vertebrate chordates have been shown to possess some cells with placode-like qualities which may be rudiments of cranial placodes. For example, Abitua and colleagues (2015) presented evidence that the neural plate border of ascidian embryos gives rise to placode-like structures, producing ciliated primary sensory cells[103]. This neural plate border region expresses homologs of many of vertebrate genes associated with the placode lineage including *Six1/2, Foxg* and *Eya* in a domain that resembles that of vertebrates and is referred to as a 'preplacodal-like' domain [103-105]. Interestingly, ascidian bipolar tail neurons, which arise from the neural plate border, can be transformed into the placode-like palp sensory cells[84]. Taken together, these data support the idea that placode cells may have evolved from the border between the neural and non-neural ectoderm and may share a common precursor with neural crest cells.

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