Neural Crest and Placodal Cells Contributions to Cranial Sensory Development

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ABSTRACT

The sensory system of vertebrates is incredibly complex. Many important components of the sensory system are located within the cranial region, including the sense organs and cranial sensory ganglia. Early in development two progenitor populations, the neural crest and the cranial placodes, arise at the neural plate border and throughout vertebrate development contribute to the developing vertebrate peripheral sensory system. The interactions and contributions of both of these cell populations to the development of the pituitary system, the eyes, the nose, the ears, and the cranial ganglia of the head and neck are vital for the appropriate development of an embryo's nervous system.

In this dissertation we explore the contributions of both the neural crest and placodal cells to the sensory system of the developing embryo. In Chapter 1 we review the origin of these two cell populations at the neural plate border and give an overview of the development of the various cranial peripheral sensory systems and their placode and neural crest contributions.

In Chapter 2 we use replication incompetent avian retroviruses to lineage trace both the olfactory placode and the neural crest to their respective cellular contributions in the olfactory system. We confirm previous studies which showed that GnRH neurons of the nose receive contributions from both the olfactory placode and the neural crest and we show that both the olfactory placode and the neural crest contribute to the olfactory neurons of the olfactory epithelium. However, neural crest alone gives rise to the olfactory ensheathing cells which are critical for neuronal migration from the olfactory epithelium to the forebrain. We also

show for the first time that the neural crest gives rise to the p63 positive horizontal basal stem cell population of the olfactory epithelium.

In Chapter 3, along with collaborators from SUNY Buffalo, we show that multipotent and functional NC cells can be derived by induction with a growth factor cocktail containing FGF2 and IGF1 from cultures of human inter-follicular keratinocytes (KC) isolated from elderly donors. They also maintained their multipotency, as evidenced by their ability to differentiate into all NC-specific lineages including neurons, Schwann cells, melanocytes, and smooth muscle cells (SMC). Notably, upon implantation into chick embryos, adult NC cells behaved similar to their embryonic counterparts, migrated along stereotypical pathways, and contributed to multiple NC derivatives in ovo. These results suggest that KC-derived NC cells may provide an easily accessible, autologous source of stem cells that can be used for treatment of neurodegenerative diseases or as a model system for studying disease pathophysiology and drug development.

Finally, in Chapter 4 we discuss future directions and experiments that I plan to pursue post-graduation. I propose to conduct a closer examination of the variants of GnRH neurons across developmental time in various representative taxa of cartilaginous fish and reptiles. Furthermore, I intend to identify and experimentally confirm a molecular regulatory region for GnRH2, the most highly conserved variant across vertebrates, within the chicken embryo. Once this regulatory region is identified, the sequence can also be used to probe the genomes of other non-model taxa. Finally, I would like to

perform lineage analysis using DiI in a non-model system to probe the embryonic origins (neural crest vs. placode) of the GnRH neurons in more ancient taxa.

PUBLISHED CONTENT AND CONTRIBUTIONS

Alison Koontz & Marianne E Bronner (2021). “Retroviral lineage tracing of olfactory placode versus neural crest contribution to the chick olfactory system.” *Developmental Dynamics.* (in preparation).

AK conceived of the project, planned the project, performed all olfactory placode injection experiments, and performed all data collection and analysis. MEB assisted with the neural crest injection experiments. Both AK and MEB contributed to the writing of the manuscript.

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SMB, GT, and SA performed all *in vitro* cell experiments and were responsible for production of induced neural crest. AK and MEB performed in ovo injections within the chicken embryo, for which AK collected and analyzed data and contributed to the writing of the manuscript along with SMB, GT, SA, and MEB.

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Chapter 1

NEURAL CREST AND PLACODES IN CRANIAL SENSORY DEVELOPMENT

ABSTRACT

The sensory system of vertebrates is incredibly complex. Many important components of the sensory system are located within the cranial region, including the sense organs and cranial sensory ganglia. Early in development two progenitor populations, the neural crest and the cranial placodes, arise at the neural plate border and throughout vertebrate development contribute to the developing vertebrate peripheral sensory system. The interactions and contributions of both of these cell populations to the development of the pituitary system, the eyes, the nose, the ears, and the cranial ganglia of the head and neck are vital for the appropriate development of an embryo's nervous system. In this chapter we cover the origins of both the neural crest and placode cells at the neural plate border of early embryos, and investigate the molecular and environmental signals that influence the early specification of the various sensory regions. We then go through each cranial sensory system and describe the relative contributions from both the neural crest and the olfactory. Finally, we investigate these molecular pathways from an evolutionary perspective, and describe the changes in molecular signaling throughout the vertebrate lineage from lamprey to chicken.

INTRODUCTION

If you look across the swath of vertebrates that currently walk, swim, and slither on the planet, it is easy to become overwhelmed by their diversity. The vertebrate lineage on the tree of life ranges from animals as large as the blue whale to those as small as a dwarf mouse. There is a diversity of body plans, survival strategies, environmental niches, and reproductive strategies. However, all vertebrates find alliance in that they all come from a single, fertilized egg contributed to by both a male and female adult. All vertebrates also rely heavily on their sensory systems to aid them in the necessities of life: survival, foraging/predation, and reproduction. For vertebrates, particularly jawed vertebrates, most of the sensory organs are found within the cranial region. This is not an accident, but instead is a characteristic that not only defines our beloved vertebrates, but also plays a critical role in how successful this branch of the tree of life is.

All of the five main senses (sight, taste, sound, smell, and touch) are located in the head. This is true across all jawed vertebrates, and is a characteristic of the vertebrate phylogenetic clade. Vertebrates are mobile predators; they require activation of multiple senses at once in order to successfully hunt or forage food. And although each organism in the clade has its own specific body plan and its own way of developing from an embryo to an adult, there are similarities in the developmental mechanisms underlying this diversity. This is the baseline for the “New Head Hypothesis”, a hypothesis which posits that the similarities in the cranial sensory system plan across vertebrates is fundamentally rooted in shared characteristics of their cranial embryonic development (Gans & Northcutt, 1983).

The vertebrate head receives contributions from a relatively few number of developmental cell populations, most notably the neural crest and the cranial placodes.

Cranial neural crest cells and the cranial sensory placodes are responsible for most of the peripheral nervous system of the head, and interactions between neural crest cells and placode cells are critical for the appropriate development of the cranial sensory system. Early in development, neural crest and placode cells are in close proximity at the neural plate border, which arises during the gastrulation stage of embryogenesis. The neural plate border is a strip of embryonic ectoderm interposed between the non-neural ectoderm and the primitive neural plate. Within this border, signals from both the non-neural ectoderm and the neural plate specify the preplacodal region and the premigratory neural crest.

Throughout neurulation, neural crest cells and placode cells remain in close association. Upon neural tube closure, however, the neural crest cells undergo an epithelial to mesenchymal transition and migrate throughout the body. Placodes, on the other hand, remain within the ectoderm as regional thickenings, then undergo invagination or ingression, becoming committed to various different placode lineages, (1) adenyhypophysis of the pituitary gland, (2) olfactory, (3) lens of the eye, (4) trigeminal ganglia of the jaw, (5) ear, and (6) cranial ganglia. Throughout the development of the sensory system, the interplay between the migratory neural crest cells and the placodes result in some of the most complex aspects of the peripheral system.

Origins of the neural crest and pre-placodal region in the neural plate border

Both the neural crest and the placodes arise from the ectoderm of developing embryos. In the early embryo, precursors to these two cell populations are likely to be intermingled at the neural plate border but then express distinct gene regulatory programming to push the cells towards their distinct fates (Martik et al., 2018).

The epiblast of the developing embryo begins to show molecular patterning during the late blastula stage. At this point, the epiblast is broken into two distinct regions: the medial future neural domain and the lateral non-neural domain (Bellairs & Osmond, 2014). The expression and/or inhibition of Wnts, FGFs, and BMPs is essential for the delineation of these different regions. In the presumptive non-neural ectoderm, expression of Wnt and BMP is high, but the expression of Wnt and BMP antagonists increases as you move medially, decreasing the levels of Wnt and BMP activity in the pre-neural region (for review, Schille & Schambony, 2017). FGF expression in the medial region is also critical for a pre-neural fate (Streit et al., 2000; Karabagli et al., 2002). Wnt, FGF, and BMPs influence the expression of a multitude of markers for both the neural and non-neural regions. For example, early expression of *Sox2*, *ERNI*, *Otx2*, and *Geminin* demarcate the pre-neural domain. Conversely, expression of *Dlx5/6*, *Msx1*, *GATA2/3*, and *TfapA* demarcate the early non-neural domain (for review, Thawani & Groves, 2020).

The interaction of the neural and non-neural ectoderm is essential for neural plate border formation and the genesis of neural crest and placode cells. Indeed, grafting experiments have shown that transplanting neural plate tissue onto non-neural ectoderm induces formation of both neural crest and placode cells (Pieper et al., 2012). Furthermore, the Wnt, BMP, and FGF signals are also critical for neural plate border induction in amniotes. Many of these signals come from the hypoblast of the embryo and appear to be involved in neural plate induction at the late blastula stage (Bellairs & Osmond, 2014). Grafting experiments in which a small piece of ectoderm along with its underlying mesoderm was transplanted into the area pellucida gave rise to neural tissue, indicating the importance of the underlying cells in induction of the neural ectoderm (Martinez Arias & Steventon, 2018). However, more

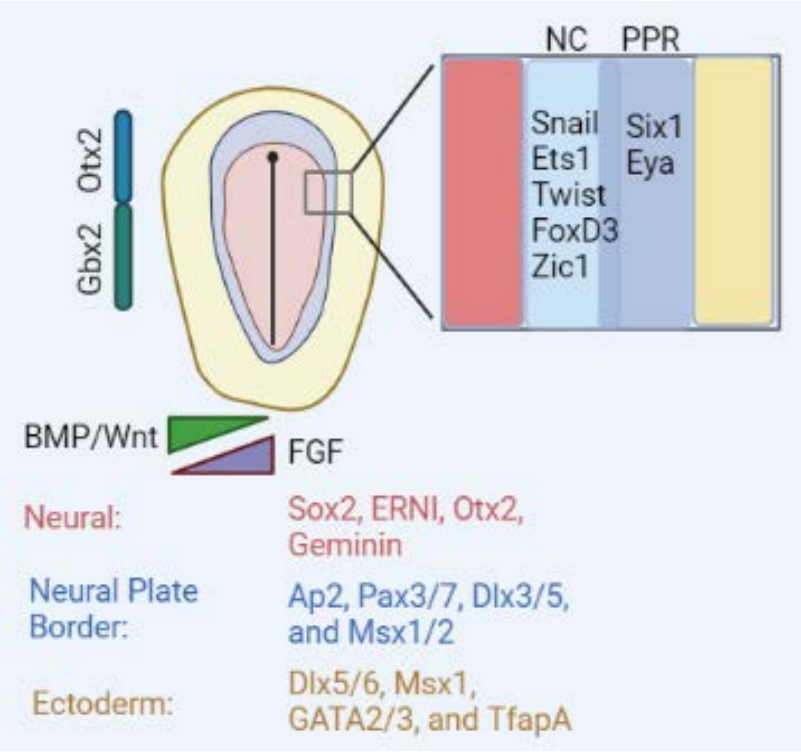

Figure 1.1. Major molecular markers in early embryogenesis and delineation of the neural plate border. Made with Biorender.

recent genetic evidence indicates that, although the hypoblast is responsible for signals that result in early neural marker expression, this expression is transient and is not itself sufficient to induce the *bona fide* neural marker *Sox2* (Albazerchi and Stern, 2007). This suggests the presence of another organizer that is responsible for later stage induction of the neural plate border, likely from the underlying mesoderm (Litsuo et al., 2005).

By the early gastrula stages, the delineation of the neural and non-neural domains is fully established, and the neural plate border begins to arise. In response to the gradiant of Wnts and BMPs, different neural plate border factors such as *Ap2, Pax3/7, Dlx3/5,* and *Msx1/2* are expressed at the medial gradient between the non-neural and neural domains (Moody & LaMantia, 2015). These expression patterns define the neural plate border region, where both the pre-neural crest and pre-placodal cells will arise.

In response to the levels of FGF, BMP, Wnt and retinoic acid, the neural plate border is subdivided into the pre-placodal region (PPR) on the lateral edge of the neural plate border and the premigratory neural crest cells on the medial border. Studies have shown that not only does the neural plate border arise at an intermediate BMP gradient, but the levels of BMP and BMP antagonists also affects the specification of the placode and neural crest cells. For example, in *Xenopus* animal cap explant experiments, higher levels of the BMP antagonist *Noggin* resulted in the expression of placodal markers, intermediate *Noggin* levels resulted in neural crest genes, and neural plate genes arose in the presence of high *Noggin* levels (Saint-Jennet & Moody, 2014).

Pre-placodal cells are delineated by relatively lower expression of BMP, and high expression of ectodermal markers such as *Six* and *Eya*. These are expressed at the most lateral region of the neural plate border (Saint-Jennet & Moody, 2014). *Six* and *Eya* are required for a PPR fate—the relative levels of *Six1* are important for determining whether a cell will become crest or placode. Overexpression of *Six1* expands the pre-placodal area at the expense of the neural crest and vice versa (Bruggman et al., 2004). EYA and SIX proteins interact, with *Eya* binding to *Six* changing the way that the *Six* transcription factor interacts with DNA (Patrick et al., 2003). FGF is also critical for the induction of the PPR: without FGF signaling, levels of Wnt and BMP signaling can expand or reduce the PPR, but FGF is required for the expression of placode markers such as *Six* and *Eya* (Litsiou et al., 2005).

The more medial section of the neural plate border gives rise to the premigratory neural crest cells, which express neural crest specifiers such as *Snail, Twist, FoxD3, Sox9, Ets1*, and *Zic1* (Khudyakhov & Bronner-Fraser, 2009). These begin to activate factors that will later be critical for the epithelial to mesenchymal transition (EMT) that allows these cells to detach from the dorsal neural tube and begin migration. *FoxD3* receives inputs from their NPB specifier genes *Pax3/7* and *Msx1* which directly bind to determine neural crest identity in the head and trunk region (Simoes-Costa et al., 2012). *Pax3/7* also directly interacts with *Zic1* and both are required for activation of *Snail* and expression of *Ets1* (Plouhinec et al., 2014; Barembaum and Bronner, 2013). The importance of *Pax3/7* and *Zic1* can also be seen in

their effects on the PPR as well. *Pax3/7* and *Zic1* expression represses the activity of important PPR factor *Six1* (Sato et al., 2010).

While the pre-placodal region and the premigratory neural crest were thought to be distinct from one another with respect to genetic expression and in location within the neural plate border (Groves & LaBonne, 2014), a recent study has shown that there is overlap in gene expression within the individual cells of neural, neural crest and placodal markers at the location where the PPR and NC meet (Roellig et al., 2017). This raises the intriguing possibility that these cells originate from a multipotent cell population with developmental potential for both neural crest and placodal fate. Previous DiI lineage analysis experiments support these findings, and found that labeled groups of cells could be found in both neural crest and placode cells, although this occurred rarely and is confounded by the labeling of multiple cells (Streit, 2002; Pieper et al., 2011). However, only single cell lineage tracing will confirm whether placodal and neural crest cells can arise from a common progenitor.

Specification of the neural crest and placodes

With the closure of the neural tube, the premigratory neural crest cells undergo EMT and become migratory neural crest cells that then delaminate from the neural tube and begin their long migration throughout the embryo and to their various derivatives. These migratory neural crest cells express *Sox10, Sox9, FoxD3, Ets1,* and other migratory markers. At the same time, there is repression of epithelial cell markers, such as *NCadherin* and *ECadherin,*

and upregulation of mesenchymal cell markers such as *Cadherin11* and *Cadherin7* (for review, Simoes-Costa & Bronner, 2016).

The pre-placodal cells, however, remain as thickened regions within the ectoderm. Various molecular markers define the ectodermal regions that will become the different placodes (Saint-Jennet & Moody, 2014). These placodes are the (1) adenohypophysis (pituitary gland), (2) olfactory, (3) lens, (4) trigeminal ganglia, (5) otic, and (6) epibranchial ganglia. Each placode is located within a distinct region of the embryo ectoderm, and therefore each placode receives different environmental signals which result in their specification.

Demarcating the beginning of the individual specification of each placode is difficult, as transplant experiments have shown that, at least for a time, the ectoderm adjacent to the neural plate is capable of giving rise to all of the different placodes. In a classic grafting experiment, the ectoderm adjacent to the neural plate was flipped on its anterior to posterior axis, so that the grafted tissue was “upside down” from its original location (Jacobson, 1963a). If the tissue had already been specified for a specific anterior placode, such as the lens or the olfactory, the transplant would not have affected the placode identity of the cells. Instead, the grafted tissue formed placodes according to their new position. However, if the same experiment was done a few hours later, the tissue developed according to its original position. This experiment shows that there is a transient period where the PPR has the

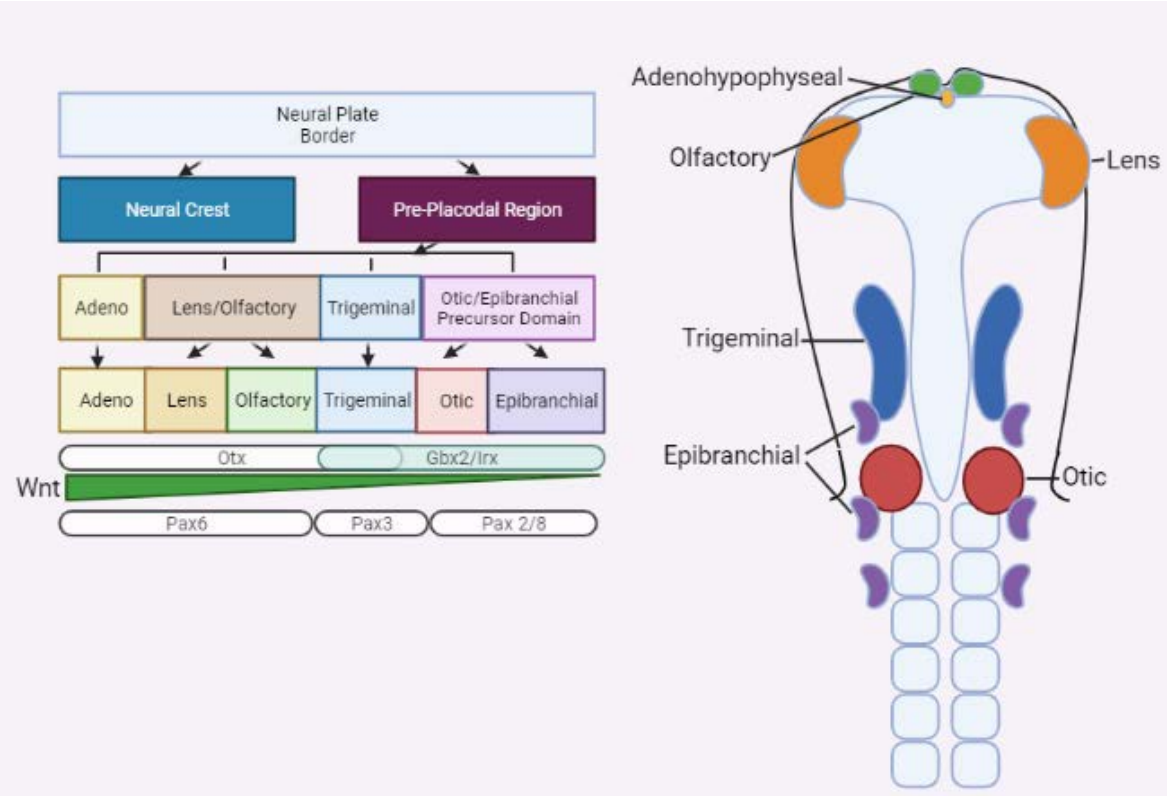

Figure 1.2: Locations of cranial placodes in HH10 chicken embryo. Lineage tree with major regionalizing molecular markers for each placode. Made with Biorender.

potential to give rise to all different placodes, but shortly after specification for individual placodes begins to occur and the cells lose their potential.

However, the first patterning that begins to define the placodes is the anterior and posterior signals that restrict the range of placode identity based on location (Saint Jeanett & Moody, 2014). Anterior placodes are those of the lens, olfactory, and adenohypophyseal, while the posterior placodes are the otic and epibranchial placodes. Interestingly, the trigeminal ganglia is defined due to its inputs from both the anterior and posterior factors due to its location. The two important anterior/posterior factors are *Otx2* and *Gbx2* respectively (Steventen et al., 2012). These two transcription factors work to mutually repress each other, the result being a clear delineation of regionalization in the embryo. Other anterior factors include *Pax6,* and *Six3/6* whereas the posterior region is further characterized by *Irx* (Schlosser, 2006).

Following anterior and posterior regionalization, signals from tissues adjacent to the placode cells work to further restrict their fate. The further restriction of the placodes is based on some of the major players we have seen before: Wnt, FGFs, and BMPs. Pax genes are also heavily implicated in placode restriction, with certain placode fates depending on expression of different Pax genes.

Low levels of BMP are required for successful delineation of the PPR in the neural plate border. However, BMP4 is also an important player in separating the olfactory and the lens

placodes, which are relatively close to each other. In vitro explant experiments of anterior neural plate border have shown that short exposure to BMP4 results in the tissue taking on an olfactory fate, whereas long exposure results in a lens fate (Sjodel et al., 2007). Therefore, BMP4 expression determines whether pre-placodal cells choose a lens or an olfactory fate, although it should be remembered that these levels are all relative to the low levels of BMP required for formation of the PPR.

Wnt levels are also important for the adoption of certain placodal fates. When Wnt is overexpressed in the *Xenopus* animal caps, the tissue adopts an otic (posterior) fate, while when FGF (a Wnt antagonist) and Wnt are overexpressed, there is an adoption of an olfactory (anterior) fate (Park & Saint-Jeannet, 2008). This experiment indicates that higher levels of Wnt push PPR towards a more posterior identity. This conclusion is supported by the fact that zebrafish which have overactivated Wnt show reduction in the size of their anterior placodes, but expanded posterior placodes (Heisenberg et al., 1996). The Wnt/Notch system is particularly important for the identity of the otic placode (Jayasena et al., 2008). The presence of higher levels of Wnt in the posterior region of the embryo activates Notch signaling, a Wnt repressor, and activates otic specific genes including members such as *Pax2/8* and *Foxi3*.

Across all studied taxa, FGFs remain an integral part of placode specification. Indeed, the levels and exposure time of different tissues to FGF signals leads to the adoption of different placode identities. This is true for differentiating the olfactory and the lens placode:

expression of FGF8 represses the lens marker *Pax6* and results in cells adopting an olfactory identity (Bailey et al., 2006). The same is true for otic vs. epibranchial identities: prolonged exposure to FGF8 promotes an epibranchial fate while the otic placode requires only a small pulse of FGF signaling (Ladher et al., 2005).

By the end of these early patterning signals, we have regions of placodal cells that are specified for a particular placode fate. The olfactory and lens are both defined by their relative levels of BMP4 and FGF8 as well as the anteriorization factors like *Otx2* and differential expression of Wnt. The posterior placodes, the otic and the epibranchial, are determined through FGF8 and Wnt signaling. The trigeminal ganglia is unique in that it adopts its fate due to inputs from both the anterior and posterior regions. For example, in addition to reliance on higher Wnt levels, the trigeminal ganglia, expression of both *Otx2* (anterior) and *Irx* genes (posterior) are important for adopting a trigeminal identity (Saint-Jennet & Moody, 2014).

Once these regions have been established, more placode specific genes are activated to further differentiate the individual placodes. Most of these changes involve the Pax family of genes. In fact, many placodes have a placode specific Pax gene, which makes them good molecular markers. Initially, multiple Pax genes assist in regionalizing the anterior and posterior regions of the embryo, with *Pax2* and *Pax8* in the posterior and *Pax6* in the anterior, and *Pax3* in between (reviewed Saint-Jennet & Moody, 2014). From there, levels of these different Pax genes are important for placode identity. High *Pax6* expression causes

formation of the lens, *Pax3* is critical for the trigeminal ganglia, and *Pax2* expression is associated with the adoption of an otic fate.

Overlap of critical transcription factors results in the eventual demarcation of placodes following the formation of the PPR. The first step of regionalization is the expression of anterior and posterior specific genes. Among these factors are familiar players: Wnt, BMP, and FGF. Different levels of these factors push cells toward a particular fate. Other more placode specific transcription factors, like members of the Pax family, work to further specify these placodal regions so that each can further develop and contribute to the sensory system.

Development of the peripheral sensory systems

Once the different placodal regions have been determined in the embryo, placodal cells can begin the important steps that bring them to final differentiation. Considering the range of senses that we have, there is quite a diversity of cell types that these cells can become. In the following section we will take each sensory system that receives contributions from both neural crest and placode and examine how the two cell populations interact and form these structures.

Adenohypophysis/Pituitary

The anterior pituitary gland is responsible for modulating the endocrine system of our body and is in charge of incredibly important functions such as reproduction, growth, and

metabolism. The gland is formed from both the neurohypophysis (the posterior pituitary gland) and the adenohypophyseal placode, which in turn receives contribution from neural crest (Sanchez-Arrones et al., 2015).

Interestingly, the adenohypophysis has some controversy surrounding its origin—some believe that it is derived from pre-neural tissue (e.g. Couly et al., 1988), whereas others believe it comes from an ectodermal placode (e.g. Cobo et al., 2001). For the sake of this review we will assume that the adenohypophysis is of placodal origin. Although the origin of the adenohypophysis remains unclear, its later development is well characterized.

The most important structure of the developing adenohypophysis is Rathke's pouch, an evagination in the roof of the presumptive mouth. The formation of Rathke's pouch constitutes the first major step in pituitary organogenesis. The next step in adenohypophyseal development is the evagination of Rathke's pouch into the oral cavity. Eventually, there is total separation of the oral cavity from the oral ectoderm, followed by a mass proliferation of cells. It is at this time that the neurohypophysis begins to interact with the adenohypophysis. Finally, the cells begin their lineage determination and cellular differentiation programs, becoming the various endocrine cells that are so critical for the pituitary system.

A number of genes play critical roles in the initiation and formation of Rathke's pouch (Larkin & Ansorge, 2017). Many of these genes were discovered in mice knockout lines

which had various degrees of forebrain and pituitary malformation. Others characterize known human disorders such as hypopituitarism and combined pituitary hormone deficiency (CPHD). However, besides basic requirements and location of expressions, the interactions of these molecules and their prospective roles in formatting the beginning of Rathke's pouch are under characterized.

Briefly, the earliest expressed adenohypophyseal markers are *Pitx2* and *Pax6,* which in chicken are expressed as early as HH10, and are located at the anterior-medial margin of the developing forebrain next to the olfactory placode (Sjodal & Gunhaga, 2008). These transcription factors remain present in Rathke's pouch throughout the entirety of development. *Tcf4* has also been shown to be present in Rathke's pouch as early as HH14 and has prolonged expression throughout posterior adenohypophysis development (Sanchez-Arrones et al., 2015). *Sonic hedgehog* (*Shh*) is expressed in the underlying mesoderm and remains in adjacent tissue to the anterior pituitary throughout development, indicating the potential of induction activity in the adenohypophysis. Indeed, the hedgehog receptor *Patched2* (*Ptc2*) is present within the developing hypophyseal placode cells. The gene for the retinoic acid producing enzyme Raldh3 is found at HH20 within the anterior part of Rathke's pouch along with the transcription factor *Lim3, Tcf4,* and *Pitx2.* The transcription factors *Isl1* and *Six3* are expressed in the posterior part, indicating these transcription factors potentially work to distinguish anterior to posterior patterning.

Information on the nature of cellular movements during formation and evagination of Rathke's pouch is also lacking. Markers for this period of Rathke's pouch formation give some clues, however, as to the molecular mechanisms of formation. BMP and FGFs both play a major role, with BMP2/4 and FGF8/10/18 all being required for Rathke's pouch formation, and later for specific cell differentiation programs (Larkin & Ansorge, 2017). *Shh* is expressed in adjacent ectoderm and ventral midbrain during this time.

Finally, the cells undergo lineage determination and differentiation to become the variety of endocrine cells characteristic of the pituitary. For example, GATA2 specifies gonadatrophin and thyrotrophic endocrine cells whereas its inhibitor POU1F1 is critical for lactotrophic and somatrophic endocrines (Larkin & Ansorge, 2017). At this point the anterior and posterior pituitary are in close interaction with the hypothalamus and form the characteristic glandular structure.

Much work still has to be done on the development of the adenohypophysis, most notably (1) a final decision as to their embryonic origins (neural vs. ectodermal) and (2) the molecular circuit that governs the development of such important structures as Rathke's pouch. These are two great avenues for further exploration within the field.

Less information is known about the contributions of the neural crest to the adenohypophysis, especially in the chicken where original quail-chick chimera experiments suggested a neural

origin of the adenohypophysis rather than placodal (Couly et al., 1988). A recent study in mice used the Cre reporter system to trace neural crest cells through pituitary development (Ueharu et al., 2017). They noted two distinct waves of neural crest cell migration into the pituitary, once on E9.5 and another at E14.5. These neural crest cells gave rise to all the hormone producing cell lineage.

Optic

The eye is one of the most beautiful derived characters seen on this planet. Even Darwin lauded its beauty in *Origin of Species*, admitting that the existence of the eye is one of the only structures that is so spectacular his readers might doubt its arising from natural selection (Darwin, 1859). The eyes are humans' main sensory structure, and we get most of our information through our ocular interaction with the environment.

It's fitting then that the optic placode is one of the most well-known and well characterized placodes. Cellular induction was first discovered by Hans Spemann in 1901 in the lens, making it a textbook example for developmental mechanisms (Spemann, 1901). Development of the optic system can be summarized in three steps: (1) the specification of the lens from lens/olfactory precursors, (2) the invagination of the lens placode to form the lens placode pit, and (3) formation of the lens vesicle, which will give rise to future lens follicles and lens epithelial cells.

The specification of the optic placode occurs at different times in different model organisms. In chicken, the optic placode is first specified during the late gastrula stage, quite early in embryonic development, along with the rostral neural plate border (Gunhaga, 2011). Much evidence shows that initially, the preliminary lens and olfactory cells intermingle in the most rostral region of the neural plate (Bhattacharyya et al., 2004). Similar to the neural plate border, a balancing act of BMPs, FGF, Wnt and Shh signaling restricts the rostral neural plate border into pre-lens/olfactory (Gunhaga, 2011).

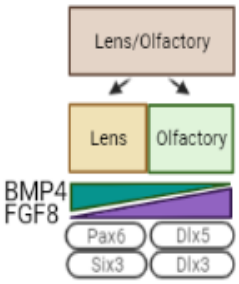

Figure 1.3. Molecular markers important for distinction of Lens and Olfactory Lineage. Made with Biorender.

Separation of the lens and olfactory begins at the neural fold stage and continues until the lens and olfactory are both morphologically distinct (Bhattacharyya & Bronner, 2008). This distinction correlates with the expression of both *Pax6* and *Dlx5*, which have overlapping expression in the neural folds at chicken stage HH8, but begin to separate spatially through migration during neural tube closure at HH10, and are separated into the lens and olfactory placode respectively by HH12-HH15, when the morphologies of both the lens and olfactory pits become visible in the embryo and the placodes are presumably committed to their fate. Interestingly, if the cells of the neural fold stage, when *Pax6* and *Dlx5* are overlapping and the olfactory and lens are intermingled, are cultured, the result is a lens fate and not an olfactory, indicating that the lens specification may be earlier and does not occur due to tissue interactions (Bailey et al., 2006).

The underlying tissues also play a role in induction of the lens placode (Cvekl et al., 2014). Signals from the underlying optic vesicle, a neural derived structure originating from the forebrain, function both to block signals originating in the lower mesenchyme from reaching the lens placode, and to instruct the lens placode through signaling of its own. BMPs are implicated strongly in the formation of the lens placode in the ectoderm. When knocked out in mice, BMP4 resulted in a loss of lens induction (Furuta & Hogan, 1998). BMP7 is also a major factor, although its exact role remains unclear. These factors activate *Sox2*, which in tandem with *Pax6* and *Six3*, an activator of *Pax6*, act in an important regulatory circuit for the committed lens placode (Cvekl et al., 2014).

Once the lens placode is committed, the next step is to form the lens pit. To do this, the ectodermal columnar cells of the placode begin to invaginate to form a pit structure. Invagination begins only following much cell proliferation and cell crowding in the placode, which is controlled in large part by *Nf1* (Carbe & Zang, 2011). Crowding triggers changes in both the cytoskeleton and the extracellular matrix of the lens placode with proteins such as Fibronectin 1 and collagen Col13al (Wolf et al., 2009; Huang et al., 2011). The action of invagination is mediated by F-actin tubulin processes in the cytoplasm, which tether the lens placode to the underlying tissue and mediate the reciprocal invagination of both the placode and the underlying optic cup (the invagination of the optic vesicle (Chauhan et al., 2009). Other proteins like Rac1 and RhoA are responsible for changing the columnar shape of the cells into more conical and apically restricted cells (Plagemen et al., 2010; Chauhan et al., 2011).

After the lens pit has invaginated, cells begin the process of differentiating into different cell types. Two of the main structures are the lens epithelium and the lens fibers. Cell cycle exit is mediated by BMP and FGF, which activate cell cycle modulators like $p27^{Kip1}$ and $p27^{Kip2}$ (Zhang et al., 1998). After cell cycle and proliferation exit, terminal differentiation can begin. The lens epithelium contains stem/progenitor cells that will continue to replenish the population of lens fibers. Expression of *Sox2* is found within these cells and is a requirement of their self-renewal capability (Arnold et al., 2011). The lens epithelium also gives rise to structural part of the developing eye that connects the lens fibers to the aqueous humor, and these cells express E and N cadherins to maintain structure, as well as beta catenin (Pontorieo et al., 2008). In fact, cataracts are caused in adults by loss of these cells.

The differentiation of the lens fibers is incredibly well studied and extensively reviewed elsewhere (see Cvekl et al., 2014). One of the major cell types that composes the lens fibers is crystalline, which gives the lens its transparency and refractive capabilities. Important cytoskeletal rearrangements such as methods of transport for water, ions, and other necessities are built between the various crystalline cells of the eye as well.

The neural crest also contributes to the development of the optic system. Although the exact nature of its contribution and interaction with the lens placode and optic vesicle is still an active area of study, it is known that following migration out of the dorsal neural tube in the cranial region, a wave of neural crest cells migrates ventrally towards the developing eye

(Williams & Bohnsack, 2015). There, it makes contact on the medial side with the optic vesicle where, through mechanisms unknown, they contribute to the optic stalk and cup of the optic system. Neural crest cells are also important in establishing patterning in the optic cup that will influence future cell development. Deletion of important neural crest factor *Sox4a* resulted in malformed optic cups and a failure of fissure closure in the eye, resulting in numerous defects (Wen et al., 2016). Recently, researchers have shown that the neural crest interaction during the formation of the optic cup is due to basement membrane epithelium and changes in the extracellular matrix (Bryan et al., 2020).

Although certainly not exhaustive, this summary should at least give a snapshot of the placodal and neural crest contributions to the developing eye. At numerous steps along the way, mutation in any of these factors or changes in cell behavior have dire outcomes for one of our most important sensory systems. Although this placode is arguably the one with the most knowledge accumulated, there is still much to uncover, for example, the cellular interactions between the neural crest and the optic vesicle that causes optic stalk formation.

Olfactory

The olfactory system is one of the prime sensory systems for many animals. Olfaction allows for the sensing of not only odors associated with survival, but also pheromones which are critical for reproduction. The olfactory system receives contribution from both the neural crest and from the olfactory placode and has some of the most diverse cell types in the cranial

sensory system (reviewed in Chapter 2). Its development is very similar to that of the lens in that the steps are (1) specifying the olfactory placode from competent pre-placodal cells, (2) invaginating to form the nasal pit, and (3) formation of the olfactory epithelium (and vomeronasal organ in some animals) and differentiation of the final olfactory cell types.

The beginning of the olfactory system is very similar to that of the lens. Briefly, the competent lens and olfactory cells intermingle at the neural plate border early in embryonic development. Initially expression of *Pax6* and *Dlx5* overlaps between the lens and olfactory placode cells in the head fold stage, but over time the region of overlap between the two is reduced until expression patterns are completely separate at commitment of the olfactory at about HH14 in chicken with *Dlx5* in the presumptive olfactory placode and *Pax6* in the lens (Bhattacharyya et al., 2004).

Other factors that are important for olfactory placode specification are *Oct-1* and *Sox2*. If these two genes are knocked out homozygously along with *Pax6*, the olfactory placode is not specified and the resulting mutant mice have no olfactory morphogenesis (Donner et al., 2006). Expression of *Dlx3* is also low throughout specification, but rises sharply following olfactory placode commitment at around HH14 in chicken.

From the thickened region of ectoderm in the presumptive nasal region, the placode must begin its invagination to become the olfactory pit. Important signals from the underlying frontonasal mesenchyme play a critical role in inducing the invagination of the pit. These

signals include retinoic acid signaling (RA), FGF, BMP, and Shh, which are used to define the mesenchymal/epithelial axes and the early patterning of the developing olfactory (Trelour, 2010). RA is a lateral signal, BMP4 is a posterior signal, and FGF8 and Shh are both medial signals, and these work to pattern the tissue to influence the invagination of the nasal pit. One study also showed that Wnt knockouts in the ectoderm led to embryos that did not form a nasal pit, further implicating this pathway as an epithelia/mesenchymal differentiator as well (Zhu et al., 2016).

During invagination, F-actin and Myosin II are responsible for the the apical constriction of cells of the epithelia as they begin to invaginate. A study from 2015 found that the activity of these proteins in placode invagination is regulated through the BMP pathway which may be providing M/E axis patterning information (Jidigam et al., 2015). As the epithelia invaginates deeper into the mesenchymal tissue, it begins to take on the structure of the olfactory epithelium, and in some animals the vomeronasal organ as well. Once these structures are in place, the cells begin to differentiate.

Within the olfactory epithelium, there are a number of different cell types including the sensory neurons, supporting cells of the epithelia, the mucus producing Bowman’s gland, and the basal stem cells which are responsible for continued neurogenesis in the nose throughout adulthood.

The lineage of the olfactory sensory neurons has been well characterized already. First, the basal stem cells produce *Mash1* positive transit-amplifying cells (Cau et al., 2002). *Mash1* is part of an evolutionary conserved mechanism that regulates neuroectodermal lineages and is important for the transition to a neuronal state. *Mash1* positive cells then go on to produce another set of transit-amplifying cells, the intermediate progenitors, which express *neurogenin 1*, a necessary factor for a variety of cell differentiation pathways, including neural. *Neurogenin 1* positive intermediate progenitors then go on to terminally differentiate into the final sensory neurons which express important genes like OMP (olfactory marker protein) and NCAM.

The basal cells of the epithelia are actually divided into two distinct populations, the horizontal basal stem cells (HBC) and the globulose basal stem cells (GBC) (Carter et al., 2004). Lineage tracing experiments have revealed that the horizontal basal cells generally remain mitotically quiescent unless activated by injury (although some studies dispute this; Iwai et al., 2008) while the globose basal cells have both reserve and active progenitors in their population with which they repopulate the neurons of the nose. Experiments *in vitro* indicate that the neurogenic differentiation potential of the GBC is regulated by BMP4 and FGF2 (Calof and Chikaraishi, 1989; DeHamer et al., 1994). Notch receptors and ligands play a large part in the signaling that influences the expression of factors such as *Mash1* and *Hes*, which are critical for later differentiation to neuronal or non-neuronal fates (Choi et al., 2018).

The HBCs on the other hand are thought to remain as a quiescent population until the destruction of sustentacular cells of the OE. During stable times, the HBCs are kept quiescent by a Notch regulatory system that maintains high expression of *p63* in the HBC so that they remain quiescent and pluripotent (Herrick et al., 2017). Only upon injury of specifically sustentacular cells does the destruction of Notch ligands cause a drop in *p63* expression and activates HBC proliferation.

Gonadotrophin producing hormone (GnRH) cells also have their origin at the olfactory epithelium. Together with the olfactory ensheathing cells, and other cells of unknown function, both OSNs and GnRH neurons send axons to the telencephalon and the presumptive olfactory bulb (OB) which are myelinated by the OECs (Perera et al., 2020). Along these axons travel olfactory ensheathing cells, and putative OMP positive "guide post" cells in a "migratory mass".

What is so curious about olfactory neuron migration is that the axons do not follow an already established migratory pathway—instead the first migratory neurons act as "pioneer neurons", setting down the track using environmental cues from both the telencephalon and the mesenchyme to reach the brain so that other neurons may follow (Whitlock, 2001). Once established, these neurons remain in the presumptive olfactory nerve layer in the forebrain as the beginnings of the olfactory nerve. A small subset of these migrate farther and more ventrally into the brain, where they are thought to stimulate the construction of the OB.

The placode contributes the most to the cell types of the olfactory system, but there are some contributions from the neural crest as well. Neural crest cells have been shown to give rise to the olfactory ensheathing cells (OECs) that myelinate the axons that olfactory neurons and GnRH neurons use to project to the olfactory bulb. There is also dispute as to whether they give rise to a subpopulation of the GnRH neurons as well. This will be reviewed extensively in Chapter 2.

Trigeminal Ganglia

The trigeminal ganglia is the largest sensory ganglia and is composed of three trigeminal nerves, the ophthalmic, the maxillary, and the mandibular, which innervate mechanoreceptors, thermoreceptors, and nociceptors and are the means by which we sense much of our head and face (Durham & Garrett, 2010). In addition to the neurons of the ganglia there are two glial cell types: the Schwann cells and the satellite glia. Trigeminal development is marked by (1) ganglia condensation, also called gangliogenesis and (2) differentiation into the various neurons and glia of the ganglia. This area is a ripe place for further research, as not much is currently known about trigeminal gangliogenesis.

The trigeminal region is demarcated early in development by overlapping expression of both the anteriorizing and posteriorizing factors *Otx2* and *Irx*. From there, numerous signals are important for gangliogenesis and for imparting a trigeminal sensory neuron identity onto cells. For instance, the factor *neurogenin 1* has been shown to not only be an important regulator of sensory neuron development, but inactivation results in a glial cell fate,

implicating some neuron-glial interactions that work in tandem to reciprocally differentiate between the neurons and their associated glia (McGraw et al., 2008).

The trigeminal ganglia receive contributions from both the neural crest and the placode and these contributions are imparted in a distal to proximal wave. First, the placode produces the neurons of the distal part of the ganglia, and lays down a structural template that the neural crest then fills in with its neuronal derivatives proximally (Steventen et al., 2014). Therefore, there are neurons from both populations in the trigeminal ganglia; the distal placode derived neurons give structure to the developing ganglia and neurons whereas the neural crest derived cells fill in proximally. Neural crest cells also produce all the glia of the trigeminal ganglia.

The presence and interactions of both the neural crest and the placode cells are critical for the condensation of the ganglia early in development—ablations in neural crest prior to this result in misshapen ganglia, and ablation of later neural crest results in disruption of trigeminal condensation (Stark et al., 1997; Gammill et al., 2006). Although little is known about the molecular process underlying trigeminal condensation, it is clear that there are requirements from both the neural crest and the placode in order for condensation to proceed normally.

Otic

The otic placode is responsible for the development of the inner ear and the numerous different cell types that comprise it, including ciliated mechanoreceptors, neurons of the

eighth cranial ganglion, the cochleovestibular nerve, and numerous structural cells (Baker & Bronner, 2001). The development of the otic placode is connected with the development of the epibranchial ganglia of the jaw and neck. In fact, the otic and epibranchial placodes maintain a common progenitor population following establishment of the PPR and subsequent axial patterning—the *Pax2* domain that commences at the mid-neurula stage delineates a common progenitor domain for both the otic and the epibranchial placodes (Ladher et al., 2010). This domain is called the otic-epibranchial progenitor domain (OEPD). Lineage labeling has shown that cells within this region are capable of giving rise not only to otic cells but also cells in the epibranchial placodes.

The OEPD is induced by paraxial mesoderm, specifically the mesoderm between the first somite and the level of the third rhombomere, which lies underneath the *Pax2* ectodermal domain. However, this induction requires some neural ectoderm is also present (Ladher et al., 2010). FGF has been implicated to be a major player in OEPD formation, particularly FGF3 and FGF19 (Schmmang, 2007). Both have expression patterns placing them in spatiotemporal proximity of OEPD induction first in the mesoderm and then in the hindbrain, and knockout of both together results in OEPD formation not taking place at all (Freter et al., 2008). In chicken, the same result happens with knockout of *Fgf8*. At present it is not known whether the individual cells at the OEPD are multipotent for both an otic and an epibranchial fate, or if there are cells that are pre-restricted to each domain. Only single cell lineage analysis will be able to answer this.

Following induction of the OEPD, the otic cells of the OEPD become committed to an otic fate in response to signals from the surrounding tissue. In particular, *Wnt8a* (*Wnt8c* in chick) is implicated in differentiating the otic cells from the OEPD in a stepwise fashion following FGF mesodermal signaling (Ladher et al., 2000). Inhibition of Wnt at this stage blocks the expression of the otic-specific marker *Soho1* but does not affect *Pax2*, indicating its role in later stage identity.

Once the otic placode is separated from the other placodal fates, it thickens into the ectodermal placode. During this period, the epithelium of the region thickens as a result of increased cell proliferation and packing within a pseudostratified epithelia. The otic placode will then begin to invaginate, and can be seen at this point as the otic pit lateral to the first couple somites. The same mechanisms of placode invagination occur within the otic as well, namely the remodeling of the cytoskeleton of the cells, including their actins, microtubules, and intermediate filaments (Whitfield, 2015). At the 10 somite stage, actin is present in both the apical and basal region of the cell. By 13 somites, actin stores have been depleted in the basal region of the cell and have been moved to the apical portion. By 16 somites, the actin of the cells is capable of forming the otic vesicles while isolated in culture.

FGF has been shown to play a role in the cytoskeletal rearrangements that precede otic invagination; indeed, introduction of FGF beads into cultures of young otic cultures (10ss) is sufficient to clear actin in the basal region of the cell (Sai et Ladher, 2008). FGF activates the motor protein myosin-II which depolymerizes F-actin on the basal side. This means that

there are two localized cytoskeleton remodeling proteins at the apical (F-actin) and the basal (myosin II) that work together to begin otic invagination. These localizations may not be restricted simply to otic invagination, but could be applied to multiple tissue invaginations across development. Interestingly, apical constriction occurs next within the otic cells, which is characterized by the localization of both F-actin and myosin II to the apical region, although the mechanisms are currently unknown.

Invagination is aided by a number of different factors which are expressed in this region at this time. For example, the transcription factor *Spalt4* is required for proper invagination of the otic placode—knockouts of *Spalt4* result in impairment of otic invagination whereas overexpression of *Spalt4* causes the creation of invaginating vesicles in non-placodal head ectoderm (Barembaum et al., 2007). In mice it has been shown that *Sox9* is required for otic invagination (Barrionuevo, 2008).

Once the otic vesicle is internalized, the two edges fuse together and surround the vesicle with overlying ectoderm. Now, the otic vesicle can begin its transformation into the final structure of the inner ear and the cells can begin to differentiate into its specialized cells (reviewed extensively in Whitfield, 2015).

Perhaps the most important cell type in the inner ear are the sensory hair cells which contain the mechanoreceptors that take external sound and convert it into electrical energy for our brain and therefore are critical for our sense of hearing. *Sox2* has been shown in many species

to demarcate the prosensory domain that is competent to give rise to these sensory hair cells, and maintenance of *Sox2* is in turn overseen by FGF signaling (Neves et al., 2013). Notch lateral inhibition also plays a large role in (1) specifying the *Sox2* positive prosensory domain and (2) supporting the sensory hair cell fate. In the chick embryo, the Notch ligands *Jag1* and *Dl1* have been shown to be differentially expressed, creating heterogeneity in the Notch signaling strength, and mediating the transition from lateral induction to lateral inhibition and the resulting preference for hair cell identity (Petrovic et al., 2014).

Atoh1 is a key player in the differentiation pathway of hair cells as it is mediated by both *Sox2* and Notch expression and is both necessary and sufficient for specification and differentiation of hair cells (Whitfield, 2015). *Atoh1* also plays a role in hair cell survival and function, along with Eps8 actin bundling proteins.

The ear is also responsible for the sense of vestibular balance and detection of your body's interaction with gravity. The cells responsible for this are the otoliths or "ear stones". Little is known about the differentiation of the otoliths in chicken, as most research has been centered around the zebrafish model. The same can be said for the formation of the semicircular canal, which senses rotation movement in the head. *Atoh1* seems to be a player in otolith formation, as knockdown of *atoh1b* results in lack of otolith seeding (Stooke-Vaughan et al., 2012). For development of the semicircular canal, researchers in zebrafish have identified *Gpr126,* a G-protein coupled receptor, that modulates the extracellular matrix of the cells that will create the canal formation (Geng et al., 2014). FGF and RA also play

roles in zebrafish as promoters and inhibitors respectively of *otx1b* which across taxa plays a role in formation of the semicircular canal (Maier & Whitfield, 2014). Mice studies have backed up the role of RA in canal formation, but also implicate Wnt/beta-catenin signaling as well.

The inner ear also includes the afferent neurons of the VIIIth cranial ganglion. These neurons are vital for our sensory hearing, as they innervate the sensory hair cells. In zebrafish, *Foxi1* has been shown to be critical for the formation of the neurons as knockdown completely removes all neuronal signaling markers from the otic region (Sai et Ladher, 2013). *Foxi1* likely plays an important role in sensory competence and neuronal specification, and its loss blocks cells of the otic from a neuronal fate. Notch signaling is also implicated in neurogenesis of the inner ear—when Notch signaling is disrupted there is an overproduction of neuroblasts.

The neural crest contributes many important cell types and structures to the ear (reviewed in Ritter, 2019). Neural crest cells are the source of the cartilage and bone for the auricle and external auditory canal of the outer ear. It is well known that defects in the neural crests ability to form cartilage can lead to microtia (reduced ears) or anotia (no outer ear). The neural crest cells that contribute to the outer ear migrate into the pharyngeal arches and these, particularly the second pharyngeal arch, gives rise to the external auditory canal. Hoxa2 is associated with microtia and impairments in cranial cartilage and bone formation and the presence of Hoxa2 is both necessary and sufficient for development of the second pharyngeal

arch (Minoux et al., 2013). Mutations in *Eya1* and *Six1* also result in malformations of the cartilage and bone derivatives of the second pharyngeal arch (Ruf et al., 2004). In fact, a study in mouse found that Hoxa2 activates *Eya1* in a subset of pharyngeal arch NC at the base of the early auricle.

In the middle ear, the neural crest contributes to the three ossicles, the auditory bulla, and the tympanic ring. The ossicles are small bones of the inner ear that serve as transmitters of soundwaves from the eardrum to the inner ear and are comprised of the stapes, the malleus and the incus. Defects in the ossicles result in hearing loss. The ossicles develop from the first and second pharyngeal arches, particularly the proximal end of Meckel's cartilage. Again, axial patterning genes such as Hoxa1 are critical for appropriate development of the three ossicles (Gavalas et al., 1998). *Tbx*, a transcription factor essential for the migration of the neural crest into the second pharyngeal arch, is also critical—knockout of *Tbx* results in malformation of the various ossicles (Moraes et al., 2005). Neural crest migration is also critical for the structure of the middle ear cavity, and neural crest cells comprise the inner epithelia of the middle ear. Furthermore, the auditory bulla and the tympanic ring are two bony structures of the middle ear that rely upon the cartilage and bone neural crest contributions to maintain their proper structural integrity.

In the inner ear, the neural crest contributes to more canonical neural crest derivatives such as the glia to the cochleovestibular ganglion and the melanocytes of the inner ear structures.

The development of the ear depends on contributions from both the neural crest and otic placodes, without which the sensory capabilities of the ear are compromised.

Epibranchial Ganglia

The epibranchial series of placodes form the geniculate, petrosal, and nodose ganglia. These ganglia contribute sensory neurons to the main cranial nerves including the VII (facial, IX (glossophyrangeal) and X (vagus). The placodes are located dorsocaudally to the branchial arches. These are the most understudied of the placodes, and thus have less published information about them.

As has been stated above, the otic and epibranchial placodes share early developmental patterning including *Pax2* expression, which distinguishes the OEPD where precursors for both the otic and epibranchial placodes reside (Ladher et al., 2010). Induction of the OEPD relies on signals from the paraxial mesoderm beneath the OEPD, but only in combination with the presence of neural precursors. FGF is also a huge contributor to the induction of the OEPD.

Unlike the otic placode, which is specified in response to signals from the mesoderm, the epibranchial placodes are induced in the OEPD by the pharyngeal endoderm (Ladher et al., 2010). The pharyngeal endoderm expresses both FGF and BMP signals which are involved in a signaling hierarchy that distinguishes the epibranchial placode. Firstly, FGF is expressed

in a broad stripe lateral to the otic placode, delineating the beginning of epibranchial specification and the zone of epibranchial competence. BMP is only expressed within the pharyngeal pouches and is therefore important for specifying the particular epibranchial placode, as each is associated with a distinct pharyngeal pouch.

Contrary to other placodes we have looked at, the epibranchial placodes do not undergo invagination. They instead remain as regions of thickened ectoderm with increased apical cell division. Neuroblasts in the basal region of the placodes migrate from the placode into their respective cranial ganglia, forming the neurons that are so vital for the cranial nerves.

Similar to the trigeminal ganglia, the migration of the epibranchial neurons into and the condensation of the ganglia is a prime example of the importance of both the neural crest and the placode cells in sensory development. The neural crest streams that are present within the pharyngeal arches actually work to guide the migrating placodal cells to their respective placode. Ablation of the neural crest in chick results in epibranchial ganglia that have misplaced axonal projections and fail to make connection with the hindbrain (Begbie & Graham, 2001a). The same happens if there is molecular perturbation of the neural crest, such as the neuropilin/semaphorin pathway (Schwartz et al., 2008).

One study of the developing taste buds of the head shows that although the epibranchial placode cells develop independently from the neural crest, they create a scaffold to support the development of the hindbrain visceral motoneurons and control the formation of neural

crest–derived parasympathetic ganglia (Coppola, 2010). However, the exact mechanisms of these migrations and the interactions of epibranchial placode neurons with neural crest is an area of further research.

Evolutionary Origins and Differences Among Taxa

How did the cranial placodes and neural crest come to be such a major player in vertebrate cranial formation? The "new head" hypothesis stipulates that the evolution of the vertebrate head is linked to the phylogenetic origin of the neural crest and the cranial placodes (Northcutt & Gans, 1983). The evolutionary pressure of food acquisition and a transition from filter feeding to mobile hunting required a far more intricate sensory system that became regionalized within the head and includes modifications to the cranial neural crest and the placodes (Northcutt, 2004). The new vertebrate body included the origin of a craniofacial skeleton, changes in pharynx structure, the regionalization of a brain into the cranial region and the appearance of novel sensory systems such as those of the peripheral nervous system. These all are important modifications for an organism that becomes adapted for hunting and foraging rather than passive filter feeding. Over time vertebrates also evolved a jaw, which involved modifications to the craniofacial skeleton and was a large aid in food acquisition.

Jawless vertebrates, such as lamprey, serve as a good outgroup for vertebrate comparison as they are one of the only extant jawless vertebrates left. Comparing this jawless vertebrate to "higher" vertebrate taxa can elucidate changes that occurred in the development and the underlying genetic program of the neural crest circuit over evolutionary time.

Protochordates, such as tunicates and cephalochordates like amphioxus provide a glimpse into potential ancestral states of pre-vertebrate organisms.

It's thought that the neural crest and placodes arose from the epidermal nerve plexus of basal protochordates (Northcutt, 2004; Patthey et al., 2014). This is supported by morphological and molecular evidence. Firstly, the GRN of the neural plate border is deeply conserved within chordates, ensuring the primary origin of both neural crest and cranial placodes are shared across species. There is also abundant morphological and molecular evidence that indicates shared characteristics between neural crest/placodes and ancestral chordate structures.

For instance, there are a few putative homologous structures within protochordates that correspond to some of the cranial placodes. Hatscheck's pit is a structure on the roof of the pharynx of amphioxus that corresponds in both location, anatomical structure, and hypothesized pituitary function to Rathke's pouch in vertebrates, a structure from the adenohypophyseal placode. Similarly, the otic placode is thought to have a homologue in the atrial syphon primordia in ascidians, which share a number of cell types like mechanosensory neurons with sensory cilia. Later, research indicated that indeed these two structures shared homologues of important otic genes *Pax2/8* that were expressed within the atrium of ascidians. Hatscheck's pit also shares expression of *Pitx* with Rathke's pouch, giving further evidence for the potential evolutionary origin of these placodes.

The neural crest is far more studied with regard to their ancestral morphological and genetic state. Although some invertebrate chordates contain subsets of neural crest function, only vertebrates have bone fide neural crest that give rise to all neural crest derivatives. For instance, there are cells within *Ciona intestinalis* and the tunicate *Ecteinascidia turbinate* that originate at the neural plate border before migrating to become pigmented sensory cells. This may indicate a cell type that predates early melanocytes, a unique derivative of the neural crest.

Perhaps the most convincing data, however, are the conservation of certain genes and genetic regulatory sub circuits specific to the neural crest across vertebrates. Much of the canonical neural crest specification cascade is conserved from lamprey to mice. However, that doesn't necessarily mean that there were no changes in gene expression in any of the species—in fact, tracking the changes in neural crest sub circuit expression offers a new perspective on viewing the molecular changes that yielded morphological results throughout the evolution of vertebrates.

A recent study dove deeper into the molecular evolution of a neural crest sub circuit throughout vertebrates. The cranial genetic regulatory circuits of the basal lamprey were compared to that of skate, zebrafish, and chicken, as examples of sequentially higher taxa (Martik et al., 2019). Previously, a cranial-specific sub circuit of the neural crest was found in chicken, with major players such as *Bm3, Dmbx1,* and *Lhx5* being important early cranial specifiers at the neural plate border, *Sox8* being present in premigratory cranial crest, and

Ets1 being important for migratory cranial crest (Simoes-Costa, 2016). The presence of these axial specific networks was not known across the vertebrate tree and it was unclear whether the present neural crest/placode GRN circuit is conserved across vertebrates, including the axial specific circuits, or whether there are divergences in expression patterns.

Lamprey, it turns out, do not have a cranial sub circuit—instead, their gene expression pattern is more similar to that of the trunk, and their premigratory and migratory neural crest do not express critical cranial factors such as *Ets1, Bm3, Dmbx1,* and *Lhx5*. What's more, genes such as SoxE, the homologue for *Sox8, Sox9,* and *Sox10,* as well as *Tfap2a* were expressed in the neural crest, although along the entirety of the body axis, indicating no specificity to the cranial region. That is not to say that the cranial specific genes like *Lhx5, Dmbx1,* and *Bm3* are not present in lamprey. They are present in the genome and in fact are expressed at later NC stages in the pharyngeal arches, which may indicate that later vertebrates co-opted already present late stage NC derivative circuits for use in earlier development.

Skates, on the other hand, do express *Ets1*, indicating an acquisition in this gene on the phylogenetic tree as we move from cyclostomes to gnathostomes. They too express crest factors such as *SoxE* and *Tfap2b* throughout all axial levels with no differentiation between trunk and cranial crests. More basal vertebrates seem to have trunk-like neural crest throughout all axial levels, indicating that specific cranial identity was only acquired later.

Zebrafish acquire expression of *Dmbx1* and *Lhx5* in their neural crest regulatory circuit and these genes are specific to the neural crest of the cranial region. However, *Sox8* was present at all axial levels, despite the fact that it is associated with cranial identity in amniotes. This presents the possibility that later NC circuits from the pharyngeal arches were co-opted for earlier NC function, and the full cranial neural crest circuit is a derived character of amniotes.

In short, development of the cranial neural crest from cyclostomes to amniotes involved co-opting regulatory modules from later neural crest development to earlier NC stages over time. You can see this clearly in the modular acquisition of cranial genes like *Ets1, Lhx5,* and *Dmbx1* across vertebrates. The ancestral state of cranial neural crest seems to be more trunk-like compared to modern amniotes, with expression of common neural crest markers present across all axial levels. The separation of cranial and trunk neural crest occurred later in the vertebrate lineage.

Much less work has been done on the GRN of placode evolution, with most focus being on early placode induction and less on differentiation into various placodes and their derivatives. There is also little information about the genetic comparisons of these placodes across taxa and through evolutionary time. This is unfortunate, as the placodes contribute just as much to the development of the vertebrate head, yet are often understudied. The evolutionary comparison of cranial placode GRNs across taxa is an intriguing area of further study.

The main takeaway, however, should be the close association of the neural crest and placodes not only temporally and spatially within embryonic development, but across evolutionary time as well. The two cell populations arose at the same time from primitive structures in the protochordates and are both critical for the evolution of vertebrates. Their shared history highlights the importance of these two structures in the development of the cranial sensory system.

CONCLUSION

Since the beginning of vertebrates, the neural crest and the cranial placodes have been extremely important during development for the building of the peripheral sensory system which allowed vertebrates to thrive. Although much is still to be learned, the origins of the GRNs that control modern development of chickens, mice, and humans is beginning to be unearthed through genomic evolution. The neural crest and cranial placodes contribute to all of the main peripheral sensory organs, including the eyes, ears, nose, cranial ganglia (such as the ones that innervate the tongue) and pituitary system. Throughout the body, the neural crest give rise to the ganglia which convey signals from our skin, such as touch, to the CNS. Although they arise at a similar location within the developing embryo, the neural plate border, from there many factors work to specify and commit competent cells into the distinct placodes and migratory neural crest. However, the interactions of the two populations throughout development are essential for appropriate development. Although this is an area still to be explored, it is becoming more and more apparent that the neural crest and placodes

work together, often times through physical interaction, to build some of the most elaborate sensory systems in the world (Steventen et al., 2014).

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Chapter 2

RETROVIRAL LINEAGE TRACING OF OLFACTORY PLACODE VERSUS NEURAL CREST CONTRIBUTION TO THE CHICK OLFACTORY SYSTEM

Adapted from:

Alison Koontz & Marianne E Bronner (2021). "Retroviral lineage tracing of olfactory placode versus neural crest contribution to the chick olfactory system." *Developmental Dynamics.* (in preparation).

ABSTRACT:

The origin of the neurons and glia in the olfactory system of vertebrates has been controversial, with different cell types attributed to being of ectodermal placode versus neural crest lineage, depending upon the species. Here, we use replication incompetent avian (RIA) retroviruses to perform prospective cell lineage analysis of either presumptive olfactory placode or neural crest cells during early development of the chick embryo. Surprisingly, the results reveal a dual contribution from both the olfactory placode and neural crest cells to sensory neurons in the nose and GnRH neurons migrating to the olfactory bulb. We also confirm that olfactory ensheathing glia are solely derived from the neural crest. Finally, our results show that neural crest cells contribute to p63 positive cells, likely to be basal stem cells of the olfactory epithelium. Taken together, these finding

provide evidence for previously unknown contributions of neural crest cells to some cell types in the chick olfactory system and help resolve previous discrepancies in the literature.

INTRODUCTION:

The olfactory system plays an important role in odorant and pheromone sensing, affecting critical animal behaviors like reproduction (Buck, 2000), inter- and intra-specific communication and recognition (Herrada & Dulac, 1997; Caro et al., 2015 for review), and acquisition of food and resources (Bertin et al., 2012). Furthermore, the ability to regenerate olfactory neurons throughout life is a property with potential applications to regenerative medicine for treatment of spinal cord and other injuries (Yuo et al., 2018 for review).

The olfactory system contains two major components: the olfactory epithelium (OE) and the olfactory bulb (OB). The OE is a sheet of cells occupying the upper regions of the nasal turbinates. Many cell types comprise the OE, the most important being the olfactory sensory neurons (OSNs) themselves, which are the neurons responsible for detection of odorants in the nasal cavity. These neurons sense odorants through their cilia, and each neuron expresses a single type of olfactory receptor (OR) that recognizes a single odorant (Buck and Axel, 1991; Ressler et al. 1993; Vassar et al., 1993). Other cell types of the OE include the supporting cells, which maintain the structure of the epithelium, the basal stem cells which are responsible for neuronal regeneration, and Bowman's gland which produces the protective mucus of the nose.

The OB is found in the telencephalon and is responsible for receiving and processing the information transmitted by the olfactory neurons in the OE. The OSNs of the OE project

axons into the OB where they make connections with glomeruli (Imai & Sakano, 2007). Each glomerulus aggregates the axons of OSNs that recognize the same receptor, and therefore each glomerulus corresponds to a single olfactory receptor (Vassar et al., 1994). The glomeruli transmit this sensory information to various parts of the brain, which then initiates a behavioral response.

Another interesting neuronal population is the gonadotrophin releasing hormone (GnRH1) neurons in the hypothalamus. GnRH neurons arise within the olfactory epithelium early in development after which they migrate out of the OE and travel through the nasal septum into the brain, before localizing within the hypothalamus (Schwanzel-Fukuda & Pfaff, 1989; Whitlock, 2005). These neurons produce gonadatrophins that are released into the pituitary system and travel through the blood stream to the gonads, playing an important role in reproductive behavior and maintenance (Casoni et al., 2016; Cho et al., 2019). In fact, mutations causing malfunction in GnRH neurons or their migration from the OE to hypothalamus result in reproductive disorders such as Kallman's syndrome (Carboni et al., 2007; Cadman et al., 2007).

All of these neuronal projections and migrations into the brain depend upon olfactory ensheathing cells (OECs), glial-like cells that myelinate axons projecting from the olfactory epithelium to the forebrain. In fact, disruption of OEC production results in failure of olfactory neuron formation and problems in axon targeting of both olfactory neurons and GnRH neurons (Saxena et al., 2013; Geller, 2013; Barraud et al., 2013).

During embryonic development, many cell populations contribute to the various cell types of the olfactory system, including ectodermal placodes and the cranial neural crest. In chick embryos, olfactory placode precursors arise in the anterior-most ectoderm at stages Hamburger-Hamilton (HH) 6-7 (Bhattacharyya & Bronner, 2004) whereas neural crest precursor are contained within the more posterior neural folds; they subsequently emigrate from the newly closed neural tube at HH9. At HH stage 10, olfactory placode precursors become specified and subsequently undergo extensive cell movement from the anterior ectoderm to the developing olfactory pit, where they are committed to an olfactory fate by HH14 (Bhattacharyya & Bronner, 2008). At this stage the olfactory pit has begun to invaginate and is visible as a region of thickened epithelium lateral to the developing lens.

Given that the olfactory system originates from different cell lineages, it is pertinent to determine the relative contributions of the olfactory placode versus the neural crest to different olfactory cell types. Previous lineage tracing experiments in various species have utilized grafting, dye-labeling and transgenic approaches, all with different advantages and disadvantages. This has led to contrasting conclusions regarding the origins of the olfactory cells (Forni & Wray, 2011). While these studies have shown a definitive contribution to olfactory sensory neurons from the ectodermal olfactory placode lineage in chicken, zebrafish, and mouse (Couly et al., 1985; Couly et al., 1987), there is evidence that pheromone sensing neurons, termed microvillus neurons, may have both a neural crest and

olfactory placode origin (Saxena et al., 2013). However, the origin of GnRH neurons, and basal stem cells remains controversial (Whitfield, et al., 2013).

Here we revisit the origin of cells in the avian olfactory system using replication incompetent avian retroviruses (RIA viruses) to label either the olfactory placode or the neural crest early in development. The use of RIA viruses for lineage labeling of chick embryos, which closely resembles human embryos at comparable developmental stages, avoids possible complications of inter- or intra-specific chimeras. We combine this lineage analysis with cell type specific labeling for olfactory sensory neurons (OSNs), olfactory ensheathing cells (OECs), GnRH neurons, and basal stem cells. The results show that both the olfactory placode and neural crest contribute to olfactory sensory neurons and GnRH neurons in birds, confirm the neural crest origin of olfactory ensheathing glia and reveal the presence of neural crest-derived p63+ cells in the OE which may represent basal cells.

RESULTS:

Recently RIA retroviruses that encode fluorescent fluorophores have been successfully used to perform lineage tracing experiments in the chick embryo (Li et al., 2018; Tang et al., 2019; Tang & Bronner, 2020). Because these viruses are replication incompetent and do not produce their own viral envelope protein, transfer of their genetically encoded information, in this case a fluorescent protein, is strictly vertical, from mother to daughter cell following cellular division (Tang et al., 2019). The RIA method of fluorescent labeling of cells is permanent, so can be used for long term lineage analysis, and is not restricted to any particular cell type, making it an elegant solution to some of the drawbacks of lineage analysis that utilize grafting or dye labeling approaches. Unlike grafting, no surgery or healing time is required and unlike dye-labeling, RIA is an indelible marker. Here, we use RIA viruses to infect either the olfactory placode or neural crest precursors in order to trace their contributions to the developing olfactory system.

Olfactory placode contributes to neurons in the chick olfactory epithelium

Previous studies that examined the embryonic origin of the various olfactory cells have come to several, contrasting conclusions across multiple developmental models (Forni & Wray, 2011 for review). Thus, the cell of origin for several olfactory cell types, including OSNs, OECs, basal stem cells (BSC), and GnRH neurons, has been a matter of debate. It was therefore our aim to probe the embryonic origins of these important cells using our novel fluorescent viruses in the chick system.

To label the olfactory placode, we introduced RIA virus encoding either H2B-YFP or H2B-RFP onto the cranial anterior ectoderm of the chicken embryo at HH10-HH13, by which time the olfactory placode cells are specified (**Fig 2.1A**). At these stages the neural crest has migrated away from the midline of the neural folds and the neural tube has fully closed in the cranial region, ensuring no labeling of neural crest cells. The exact stage of infection did not significantly affect the results, although earlier stages tended to have a higher number of infected cells compared to older stages. Therefore, we focused our injections at HH10-11. Embryos with labeled olfactory placode cells were allowed to develop for 5- 7 days post injection, harvested and fixed at Hamburger-Hamilton stages HH29 (N=7), HH31 (N=3), and HH34 (N=8). Whole mount imaging of the craniums of labeled embryos revealed ample infection of the nasal region (**Fig 2.1B-C**). Sections through the nasal region of the embryo 7 days post injection revealed virally labeled cells in the OE, as well as in cells migrating from the OE to the olfactory bulb, and in the OB in all injected embryos (**Fig 2.2**). Of note, this labeling method only marks a subset of olfactory placode cells or neural crest cells rather than the whole population.

To quantitate the efficiency of infection, we generated frontal 30μm sections from the frontal and medial region of the nasal turbinates and the OEs of representative HH34 embryos (n=6 olfactory placode) and compared viral numbers with HuC/D staining (**Table 1**). As the number of infection events per embryo varies, counts of the number of virally labeled cells throughout the OE varied widely with the olfactory placode embryos averaging 78 cells (± 25 SEM; n=15). Whereas some slides contained numerous (over 400)

virally labeled cells, others were only sparsely labeled with less than 10 YFP expressing cells. This variability is likely due to the random initial infection rate of the embryonic ectoderm coupled with and differing concentration of the RIA virus between experiments. We noted ample infection within the naris of the developing olfactory system (**Fig 2.1B-C; Fig 2.2**). Furthermore, overlapping expression of virally expressed fluorescent protein and the neuronal marker HuC/D confirmed that the virally labeled olfactory placode cells gave rise to the olfactory sensory neurons of the naris in all experimental embryos sectioned and examined at HH34 (n=15; **Fig 2.2B-F**). In the olfactory placode embryos, the average number of HuC/D/RIA double positive cells per slide was 31 (± 11 SEM; n=15) and 35% (±7.5 SEM%; n=15) of all the virally labeled cells were co-labeled with HuC/D.

Neural crest contributes to neurons in the chick olfactory epithelium

To label a portion of the neural crest population that will contribute to the olfactory system, we injected RIA virus encoding H2B-YFP into the closed neural tube at the level of the developing hindbrain in HH9 embryos such that the tube filled from posterior to anterior to the level of the caudal forebrain. During this stage, neural crest cells at the level of the forebrain and midbrain are just beginning to undergo an epithelial to mesenchymal transition to become migratory neural crest cells, after which they will exit the neural tube and migrate throughout the embryo. We found that injection into the neural tube at this time point resulted in infection of cranial neural crest prior to and during migration (**Fig**

2.1D-F). Embryos were allowed to develop for 6 days post injection (HH30), before being collected and sectioned (N=10). To rule out any placode labeling from virus possibly escaping through the anterior neuropore, embryos that exhibited labeling in the anterior ectoderm, particularly around the eyes or nose, were excluded and injections in most embryos were confined to the caudal forebrain/midbrain axial level.

To quantitate the efficiency of infection, we generated frontal 30µm sections from the frontal and medial region of the nasal turbinates and the OEs of representative HH30 neural crest embryos (n= 5) and compared viral numbers with HuC/D staining. As the number of infection events per embryo varies, counts of the number of virally labeled cells in the neural crest embryos averaged 51 (±16 SEM; n=11). The majority of neural crest labeled embryos exhibited some labeling in the olfactory epithelium (N=8/10). Five of these embryos had labeling in the anterior HuC/D positive portion of the olfactory epithelium while the others only had positive cells in the non-neurogenic posterior region of the nostril (**Table 1**). A large percentage of virally labeled cells co-labeled with the neuronal marker HuC/D (**Fig 2.3B-D**). In the neural crest embryos, the average number of HuC/D double positive cells per slide was 12 (± 3.0 SEM; n=11) and 42% of (± 11 SEM%; n=11) of all virally labeled cells co-labeled with HuC/D. indicating that many of the other H2B labeled cells in both the olfactory placode and neural crest embryos contribute to other cell types of the olfactory epithelium as well as immature neurons.

Furthermore, viral infection was not only noted in the olfactory naris, but was also observed in the developing nostril (**Fig 2.2A; Fig 2.3A**). These data indicate that neural crest cells give rise not only to olfactory neurons, but also to supporting cell types of the naris. In the neural crest embryos, many YFP positive cells were also found clustered around the developing turbinates within the developing naris (**Fig 2.3A**). These cells are likely either neural crest cells that contribute to the craniofacial cartilage and bone of the nasal region, as evidenced by their cobblestone-like morphology characteristic of cartilage, or olfactory ensheathing cells that are preparing to ensheathe olfactory axons once they begin to migrate.

Previous work in zebrafish has suggested that some microvillus neurons of the olfactory epithelium are neural crest-derived. By staining with TRPC2 antibody, a common marker for microvillus neurons in other species, we failed to identify microvillus neurons in the chick olfactory epithelium. This is consistent with reports suggesting that chick may lack this cell population. However, this is a negative result and therefore it remains unclear whether birds have microvillus neurons or not.

Olfactory Ensheathing Cells in the migratory stream are derived from the neural crest whereas GnRH come from both placode and neural crest

GnRH neurons were originally described as originating from the olfactory placode, but later it was proposed that a subpopulation of GnRH neurons may originate from the cranial neural crest population in multiple vertebrates (Wray, 1989; Yamamoto, 1996; Whitlock, 2003; Saxena et al., 2013). Since then, a dual origin of GnRH cells has been reported in mice (Forni et al., 2011). Still others have reported a uniquely placodal origin for the GnRH neurons (Sabado, 2012).

As Sox10 marks glial cells in the developing chicken at the stages examined, Sox10 positive cells associated with migratory GnRH positive neurons were considered to be potential OECs. We examined the degree of co-labeling of RIA with GnRH and/or Sox10 antibodies in the migratory stream or olfactory bulb in neural crest injected (N=10) embryos versus olfactory placode (N=3) injected embryos in frontal 30μm sections of nasal turbinates and forebrain of representative HH34 embryos.

The cell composition of the migratory streams varied in each slide with approximately 49.0 (±7.0 SEM; n=13) total GnRH neurons and 103.3 (±22.5 SEM; n=15) Sox10 positive cells across six embryos (N=6). Within the migratory streams, the number of virally labeled cells varied from embryo to embryo. In the neural crest injected embryos, there were an average of 10 labeled H2B-YFP cells per slide (± 2.8 SEM; n=17) ranging from 2 to 39 H2B-YFP positive cells on a slide. YFP positive cells that co-labeled with Sox10 were found in four out of ten neural crest labeled embryos and an average of 22% (±4.0 SEM%, n=17) of all H2B positive cells on a slide were double labeled with Sox10 (**Fig 2.3E-H)**.

However, these double labeled Sox10 cells made up a small portion (1.7% ± 0.73 SEM%; n=8) of the total Sox10 population of the migratory streams suggesting that our lineage labeling captured a subpopulation as opposed to the total neural crest population. YFP cells that were double labeled with GnRH neurons were found in five of ten neural crest embryos (**Fig 2.3I-M**). These GnRH neurons were relatively rare and only 1 to 3 YFP cells overlapped with GnRH per slide. This suggests that the neural crest contributes a small number of GnRH neurons to the migratory stream.

In contrast, no olfactory placode labeled embryos exhibited any overlap with Sox10 positive cells in the migratory streams (N=8) (**Table 2**) but instead H2B-RFP labeled cells exhibited ample co-expression with GnRH neurons of the migratory streams (**Table 3**). RFP positive cells that co-labeled with GnRH were found in all three olfactory placode labeled embryos and an average of 30% (±7.7 SEM%, n=3) of all H2B positive cells were double labeled with GnRH making up 10 % (±2.4 SEM%; n=4) of the total GnRH population of the migratory streams.

In chick, we observed GnRH neurons emerging from both RIA-labeled ectodermal placodes and neural crest cells. In contrast, the neural crest alone gives rise to OECs in both the migratory streams and hypothalamus of chicken embryos.

Neural crest contributes to p63+ which may be basal stem cells

The basal cells of the epithelia are divided into two distinct populations, the horizontal basal stem cells (HBC) and the globulose basal stem cells (GBC) (Carter et al., 2004). Lineage tracing experiments have revealed that the horizontal basal cells generally remain mitotically quiescent in the adult unless activated by injury (although some studies dispute this; Iwai et al., 2008) while the globose basal cells have both reserve and active progenitors in their population with which they repopulate the neurons of the nose. The HBCs on the other hand are thought to remain as a quiescent population until the destruction of sustencular cells of the OE. During stable times, the HBCs are kept quiescent by a Notch regulatory system that maintains high expression of p63 in the HBC so that they remain quiescent and pluripotent (Herrick et al., 2017). Only upon injury does the destruction of Notch ligands cause a drop in p63 expression and activates HBC proliferation.

Recent experiments using P0Cre/EGFP mice indicated a population of neural crest derived cells remains in the olfactory epithelium as the HBSC (Suzuki et al. 2012). These cells can be identified using antibody staining against p63. However, no other experiments have been done to investigate a neural crest contribution to the basal cells of the olfactory epithelium.

To see if an analogous population is present in the chick olfactory epithelium. we stained neural crest labeled olfactory epithelia using antibody against p63. We found ample labeling of the caudal nostril, which may represent a population of HBSCs. Many YFP labeled neural crest cells also expressed p63, consistent with a potential neural crest

contribution to the HBSC population in the olfactory epithelium (**Figure 2.5**). One caveat, however, is the role of p63 in HBSC has only been characterized in adults and not embryonic tissues.

Olfactory placode contributes GnRH neurons in the Olfactory Bulb:

Migration of the GnRH neurons and their OEC's is critical for the appropriate development of the embryo. There are numerous factors that aid the GnRH migration, and it is during this migration that most developmental disorders arise (for review Wray, 2010). The arrival of the GnRH neurons to the olfactory bulb and the hypothalamus is critical for normal development. Once the GnRH neurons reach the olfactory bulb, they can begin their migration to their final destination in the hypothalamus.

At 7 days post injection (HH34) some of the earlier migratory streams of the GnRH migratory mass have reached the olfactory bulb (**Figure 2.4D-G**). In the more proximal region of the olfactory bulb, they group into bundles of GnRH neurons and OECs, likely also interacting with the olfactory neurons they traveled with. Probing deeper into the brain, GnRH cells and their OECs begin to enter the brain and move closer and closer towards the hypothalamus (**Figure 2.4A-C**).

We examined 30 μm frontal sections of the forebrain of two placode labeled embryos. The average number of GnRH and Sox10+ cells in the olfactory bulb were 42 GnRH neurons

(±6.6 SEM, n=10) and 216 Sox10 cells (±64.4 SEM; n=4) respectively. The placode contributed significantly to the GnRH populations of these samples. Each sample had an average of 13 YFP or RFP labeled cells (±3.7 SEM; n=12). Of that, 47%(±13.5 SEM%; n=6) of the labeled placode cells co-labeled with GnRH.

Taken together, our data show that some GnRH neurons from both the migratory streams and in the olfactory bulb originate from the olfactory placode. Surprisingly, some GnRH neurons also originate from the cranial neural crest, in support of other recent studies (Barraud et al., 2010). Sox10 positive OECs, on the other hand, come exclusively from the neural crest in the migratory streams.

DISCUSSION:

Our results show that RIA viruses can be applied to trace the lineage contribution of various cell types of interest to the developing chick olfactory system. We find that both the nasal placode and neural crest contribute to olfactory sensory neurons within the olfactory epithelium and to GNRH neurons migrating to the olfactory bulb. In contrast, neural crest cells alone give rise to the OECs in the olfactory system, confirming previous results from grafting experiments. Neural crest cells also give rise to p63+ cells within the olfactory epithelium that may represent a basal cell population. Our results also confirm these origins using a non-grafting approach, as opposed to previous studies which utilized quail-chick chimeras and transplantation of GFP-labeled neural folds (Barraud et al., 2010).

The classical model in the chicken system for lineage tracing is the quail-chick chimeric transplant experiments (Le Douarin, 1999). The drawbacks of these transplants, however, are potential species differences in the behavior of quail and chick cells during development, healing time, as well as the potential for human error during the transplantations, and the chance that more than just the targeted tissue is transplanted (Tang & Bronner, 2020). While using GFP transgenic chicks grafted into wild type hosts alleviates potential complications of interspecific recombinations, there still may be issues caused by grafting itself (Chapman et al., 2005). Our RIA viral lineage analysis has the advantage of being non-invasive and species specific to the chicken, obviating several of these drawbacks.

The origin of neurons in the olfactory system has been controversial, with different results emerging from studies in different species and using different techniques. In mice, most studies utilize a Cre mouse with an ectodermal specific enhancer of TFAP2a to drive Cre transgene expression within the placodes (Forni et al., 2011). The drawback is not only its restriction to genetic model systems, but also the potential for leaky lines and ectopic expression caused by tamoxifen addition (Song & Palmiter, 2018). Transgenic lines are also common in lineage tracing experiments in zebrafish, but are subject to similar drawbacks as the Cre/Lox system in mice (Whitfield, 2013; Saxena et al., 2013). In chick and zebrafish, many classical lineage tracing experiments utilized single cell injections of vital dye (Bronner-Fraser & Fraser, 1988; Bronner-Fraser & Fraser, 1989; Whitlock et al., 2004). This, however, restricts the analysis to the short term as the dye dilutes with each cell division, allowing only a brief period for visualization that would not be amenable to other model systems (Tang et al., 2020).

Our study aimed to use a novel technique, fluorophore encoded RIA viruses, to address lineage questions in the olfactory system using a new methodology. Our results decisively confirm the results of grafting experiments in chick and Wnt1-cre lineage tracing in mice that suggested that the neural crest gives rise to all the OECs of the olfactory system (Barraud, 2010; Forni et al., 2011). OECs initially were thought to be an olfactory placode derived cell type (Chuah, 1991; Ramon-Cueto & Avila, 1997). However, subsequent experiments showed that the OECs are derivatives of the neural crest, and that previous experiments were confounded by the close association of the OECs and olfactory cell types

(Barraud, 2010; Forni et al., 2011; Katoh et al., 2011). A neural crest origin is also supported by the fact that all previously described glial cells originate from the neural crest (Simoes-Costas & Bronner, 2015).

We find that GnRH neurons receive a majority of their cells from the olfactory placode, although the neural crest contributes a small portion of cells to the GnRH. These findings support some previous studies (Yamamoto et al., 1997; Forni et al., 2011) but contradict others (Sabado et al., 2012). Because the anterior neuropore is open at the stages of injections, we were very careful to inject virus only to the level of the forebrain/midbrain border so as not to label ectodermal cells. For this reason, we labeled at early stages of neural crest migration and these time points may miss some of the earliest migrating neural crest cells. Thus, it is worth noting the RIA only labels a subset of the neural crest or olfactory placode but not the whole population. Although the RIA approach has many advantages, it is complementary rather than a replacement for other methods of lineage analysis. Moreover, access availability of markers for different types of olfactory neurons within the olfactory epithelium make it hard to parse whether neural crest and placode cells contribute to the same types of neurons or different subsets. This will be an interesting question to explore in the future as more markers of neuronal subtypes become available.

Understanding the developmental lineage of embryonic cell populations is critical for understanding developmental disorders and diseases. OSNs are required for appropriate processing of odorants, and are critical for scent based behaviors (Buck, 2000). GnRH

neurons are required for maturation to adulthood and the onset of puberty (Casoni et al., 2016; Cho et al., 2019). Problems occurring during GnRH neuron migration from the OE to the brain can lead to diseases such as Kallman's syndrome and even sterility (Cariboni et al., 2007; Cadman et al., 2007). OECs are critical for successful GnRH and olfactory neuron migration, and without them projection of the neuronal axons that GnRH and olfactory neurons use to travel to the forebrain is disrupted, and these neurons cannot successfully infiltrate the forebrain (Saxena et al., 2013; Geller, 2013; Barraud et al., 2013). Despite their importance, however, relatively little is known about the lineage progressions that shape and define different cell types, particularly the OECs, which are not a uniform population (Yao et al., 2018). Although recent publications have added knowledge to the molecular landscape of OECs, there is much more to be discovered (Perera et al., 2020). OECs are not only critical during olfactory development, but are also important for spinal cord regeneration in regenerative medicine due to their persistence into adulthood and their ability to add in the formation of incredibly robust axons (for review, Ekberg and St. John, 2014). Understanding the embryonic developmental pathways of these cells will inform future endeavors for culturing OECs and applying them in a clinical context.

In summary, our data inform upon the relative contributions of the olfactory placode and the neural crest to the chicken olfactory system. The results show that the olfactory placode gives rise to the olfactory sensory neurons of the nose, as well as to a subset of GnRH neurons migrating to and within the olfactory bulb and hypothalamus. Neural crest cells, on the other hand, are the sole contributor of the glial-like olfactory ensheathing cells and

also contribute to OSNs and GnRH neurons. We also show that neural crest positive cells give rise to p63 positive in the olfactory epithelium that may reflect basal stem cells. These findings contribute to current knowledge of olfactory development and the developmental potentials of neural crest and placodal populations.

MATERIALS AND METHODS:

Plasmid Construction

RIA and viral vectors were modified by introducing unique AscI and NotI digestion sites to facilitate cloning (Li et al., 2018). For lineage analysis, H2B-YFP and H2B-RFP were cloned into RIA vector.

Viral Concentration and Injection

Recombinant RIA plasmids were co-transfected with Envelop A plasmid into DF1 cells in 10 cm dishes. 24 hours later, the cell culture medium was collected, and collection was repeated every 24 hours for three days. The combined medium from multiple collection days was combined and virus concentrated at 26,000 rpm for 1.5 hr. The pellet was dissolved in minimal volume of DMEM and stored at -80C.

Concentrated RIA virus diluted in a 1:1 ratio with Ringer's solution was loaded into a thin pulled glass needle pipette. For olfactory placode injection, virus was injected onto the cranial ectoderm of Hamburger-Hamilton Stage 10—12 chick embryos. For neural crest

injections, virus was injected into the closing neural tube at midbrain and hindbrain levels through the opening in the hindbrain of Hamburger-Hamilton Stage 9 embryos. Any viral leakage from the anterior neuropore was quickly washed away with Ringer's solution, mitigating viral infection of ectoderm. Embryos were covered with sterile surgical tape, and incubated at 37°C for 1—7 more days, after which the surviving embryos were dissected out, fixed with 4% paraformaldehyde in PBS for 30 min 4°C and washed 3 times with PBS.

Fixed embryos were embedded in gelatin and sectioned into 30 µM transverse sections on a cryostat. These sections were examined under a fluorescent apotome microscope (Zeiss Axioscope 2 and Zeiss ApoTome.2) for viral fluorescent signal.

Immunofluorescence

Sections were blocked with either a 10% goat or 10% donkey serum solution in PBS-Tween 0.2%, and antibodies were added to the same blocking solution. Immunostaining was performed on 30 µM sections with the following antibodies: : for Olfactory Sensory Neurons, HuC/D (Invitrogen / molecular probes 16A11 1:250); for Olfactory Ensheathing Cells, Sox10 (Santa Cruz Biotechology SC365692 1:100); for GnRH neurons, anti-GnRH1 (US Biological Life Sciences 140531 1:250); for horizontal basal stem cells, anti-TP63 (MyBioSource Cat# MBS821026 1:100); for microvillus neurons, TRPC2 (Alomone Labs Cat #: APC-045 1:100); for anti-RFP(rabbit 1:100) (MBL Cat#PM005); for anti-RFP IgG1 (Thermofisher Scientic Catalog # MA5-15257 1:100); for anti-GFP (1:100); DAPI

(1:1000). Secondary Alexa Abs (Molecular Probes) were used 1:1000. Slides were imaged using fluorescence microscopy (Zeiss Axioscope 2 and Zeiss ApoTome.2).

Quantitation:

30 µM transverse sections were stained with immunofluorescent antibodies and imaged using fluorescence microscopy (Zeiss Axioscope 2 and Zeiss Apotome.2). The olfactory epithelium, migratory streams and olfactory bulbs were looked at for 7 olfactory placode labeled embryos and 10 neural crest labeled embryos. For each structure, a minimum of 3 images were taken per embryo, with the exception of the elusive migratory streams which we took images of all examples found in section. Images were taken as a z-stack composite of 18 slices throughout the section. Images were then uploaded into Fiji software. Cell counts were done by hand using the cell counter plugin in Fiji.

For each image, the number of H2B labeled cells was counted, as well as the number of antibody stained cells (ex. anti-GnRH), and the number of cells that were labeled with both. All calculations were performed in Microsoft Excel 2016. Our cell counts and percentages are presented as an average across all slides (either placodal or neural crest) followed by the standard error of the mean and the number of sections used for the calculation.

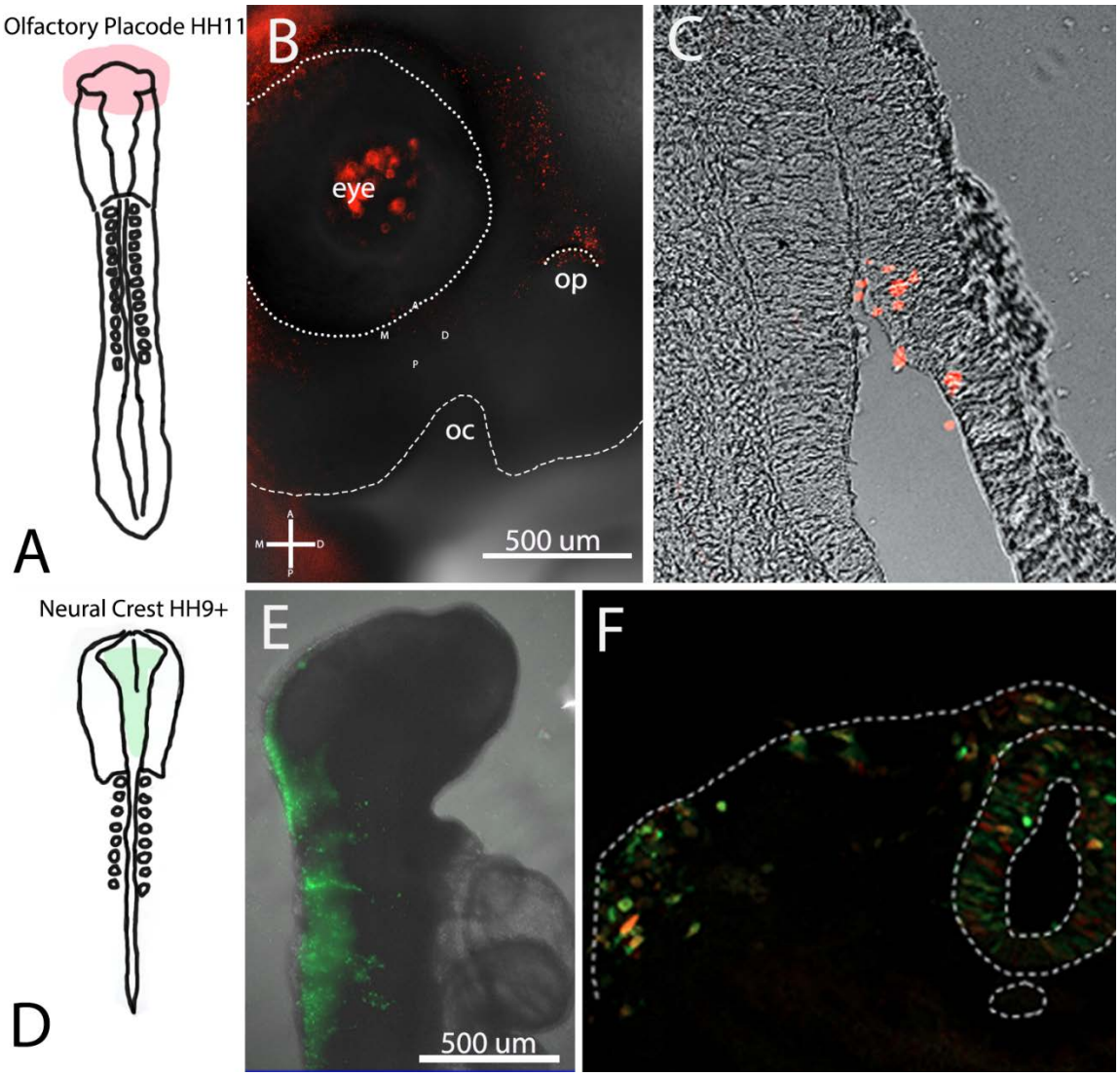

Figure 2.1 RIA Viral Injections Label the Cranial Neural Crest and the Olfactory Ectoderm (A) HH11 embryos have H2B-RFP encoding virus dripped onto their anterior ectoderm to label the ectodermal olfactory placode. (B) 2 days post injection, you can see the virus has infected the ectoderm of the head, as well as the olfactory pit (op); oral cavity (oc). (C) A section through a 3 day old embryo shows labeling of the cells of the olfactory pit, which has begun to ingress to form the olfactory epithelium. (D) HH9+ embryos have H2B-YFP encoding virus injected into their developing neural tubes, with the injection only preceding to the levels of the midbrain so as not to accidentally label cranial ectoderm. (E) 1 day post injection, you can see ample labeling of the neural tube, as well as the migratory neural crest cells. (F) A section through the embryo in (E) shows heavy labeling of the neural tube, but also labeling of migratory neural crest cells as the migrate away from the midline.

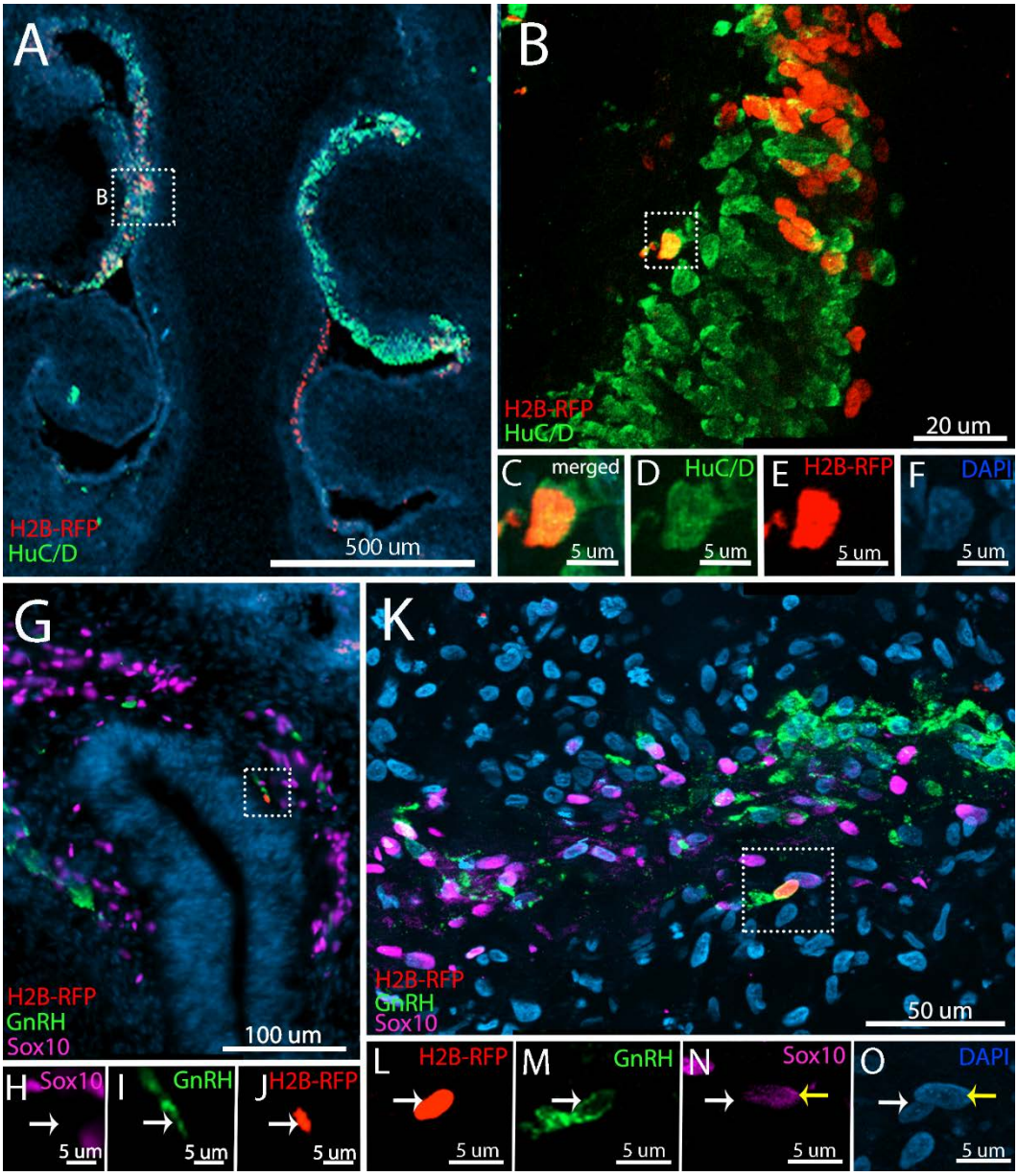

Figure 2.2 RIA-injected olfactory placodes yield multiple cell type derivatives. (A) 5x image of the olfactory naris in 7 day old chicken embryos that have had their olfactory placodes injected with H2B-RFP encoding RIA virus. Staining for the neuronal marker HuC/D reveals the presence of olfactory neurons in the anterior region. White dotted box indicates location of (B). (B) Image zoom of the olfactory epithelium of (A) containing many HuC/D positive olfactory neurons and H2B-RFP labeled cells. (C-F) An olfactory neuron double labeled with the HuC/D and virally introduced H2B-RFP. (G) The migratory stream of GnRH positive cells with their Sox10 positive OECs migrating away from the anterior-most olfactory epithelium. White dotted box indicates location of (H-J). (H-J) A GnRH neuron that is double labeled with virally introduced H2B-RFP. (K) A migratory stream of GnRH neurons and their Sox10 positive OECs traveling from the OE to the forebrain. White dotted box indicates location of L-O. (L-O) A GnRH neuron that is double labeled with virally introduced H2B-RFP (white arrows) and a Sox10 positive OEC that is not (yellow arrows).

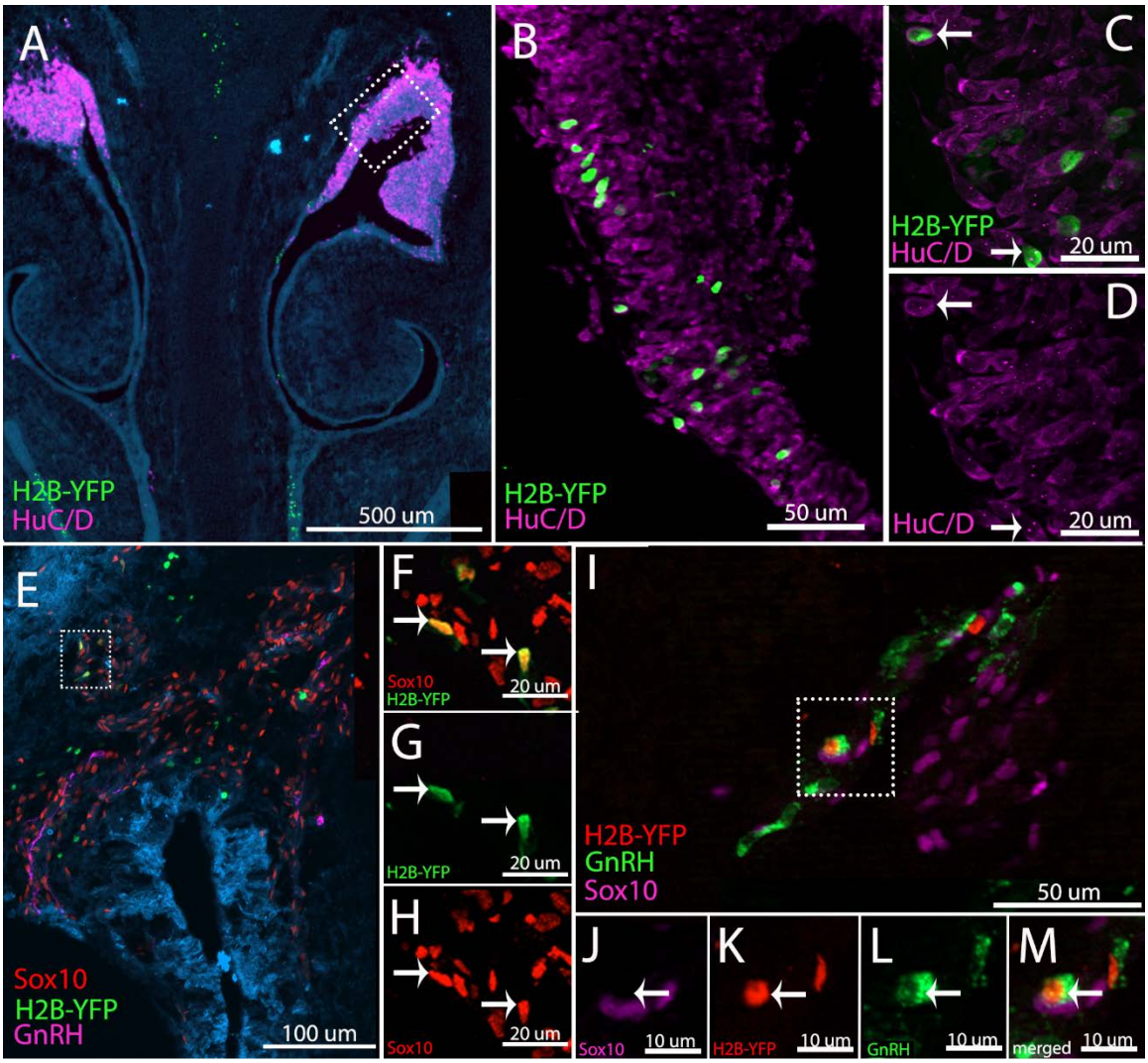

Figure 2.3 Neural crest labeled with virally introduced RIA-H2B-YFP give rise to olfactory ensheathing cells. (A) 5x image of the olfactory naris in 7 day old chicken embryos that have had their neural crest cells labeled with H2B YFP encoding RIA virus. Neural crest labeled cells are found within the HuC/D positive region of the olfactory epithelium. 5x image of the olfactory naris in 7 day old chicken embryos that have had their neural crest cells labeled with H2B YFP encoding RIA virus. Neural crest labeled cells are found within the HuC/D positive region of the olfactory epithelium. (B) The olfactory epithelium of H2B-YFP labeled NC embryos with YFP overlapping with HuC/D White box indicates locations of (C-D). (C-D) Zoomed images showing double labeled YFP neural crest cells with neuronal marker HuC/D. (E) Migratory streams of GnRH neurons and their OECs as they migrate from the

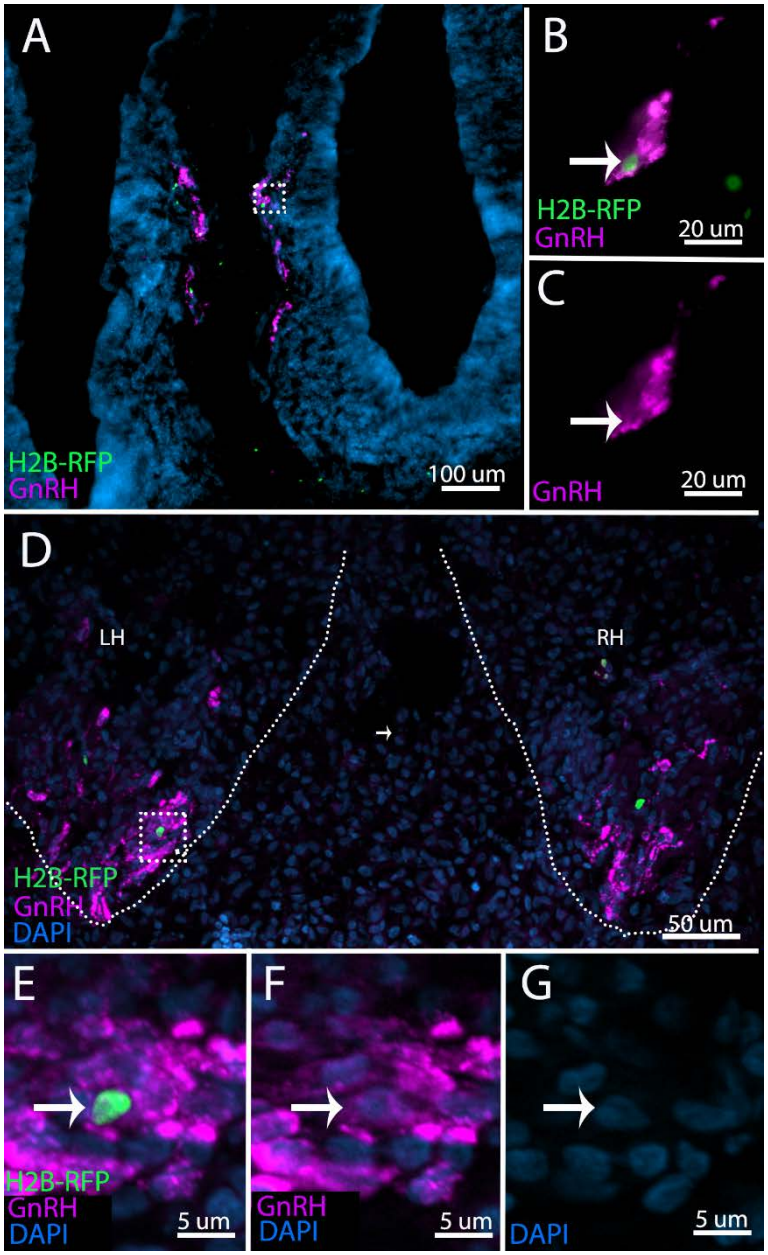

Figure 2.4 RIA-injected olfactory placodes give rise to GnRH neurons in many regions of the developing brain (A) The hypothalamus of a 7 day old chicken embryo stained with GnRH antibody. (B, C) Zoomed in image of a GnRH neuron that is double labeled with virally introduced H2B-RFP. (D) The developing olfactory bulb of a 7 day old chicken embryo showing the GnRH positive neurons that have migrated there.; left hemisphere (LH); right hemisphere (RH) (E-G) A cluster of GnRH positive neurons in the olfactory bulb, one of which is double labeled with virally introduced H2B-RFP.

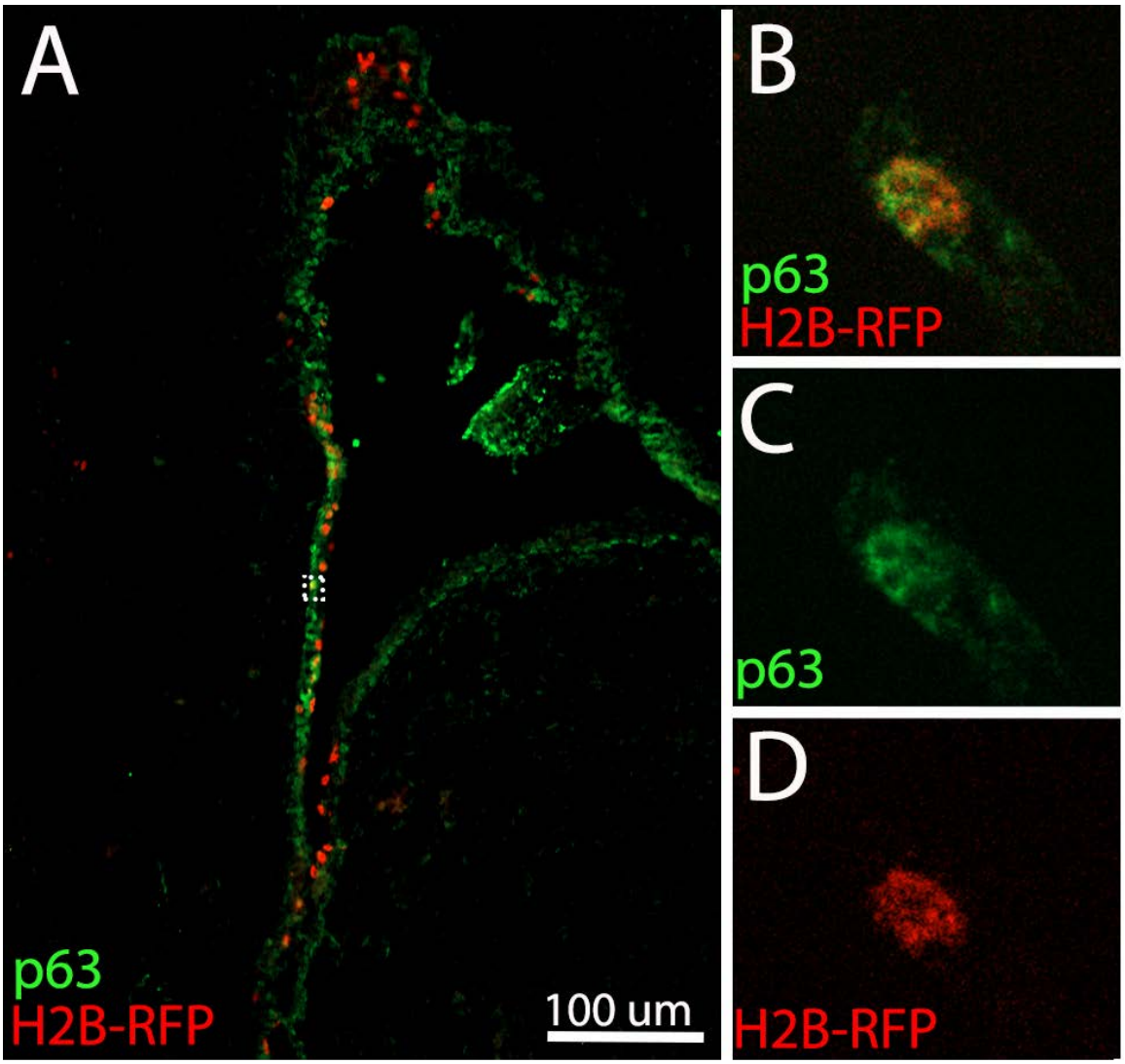

Figure 2.5 RIA-injected neural crest cells overlap with p63 positive putative basal stem cells (A) 10x image of nostril stained with anti-p63 antibody, white box indicates location of (B-D). (B-D) close up images of p63 double positive cell.

Table 1: Olfactory Placode and Neural Crest Contributions to Olfactory Epithelium

OLFACTORY EPITHELIUM	Average # of Virally Labeled Cells	Average # of HuC/D+/FP+ Cells	% of viral cells that are HuC/D+
Olfactory Placode	78 cells (± 25 SEM; n=15)	31 (± 11 SEM; n=15)	35% (±7.5 SEM%; n=15)
Neural Crest	51 (±16 SEM; n=11)	12 (± 3.0 SEM; n=11)	42% of (± 11 SEM%; n=11)

Table 2: Olfactory Placode and Neural Crest Contributions to Sox10+ OECs of Migratory Streams

MIGRATORY STREAM	Average # of Virally Labeled Cells	% of viral cells that are Sox10+	% of total Sox10+ co-labeled with virus
Olfactory Placode	9 (±2 SEM; n=8)	0% (n=7)	0% (n=7)
Neural Crest	10 (±3 SEM; n=17)	22% (±7.5 SEM%, n=17)	1.7% (± .5 SEM%; n=11)

Table 3: Olfactory Placode and Neural Crest Contributions to GnRH Neurons of Migratory Streams

MIGRATORY STREAM	Average # of Virally Labeled Cells	% of viral cells that are GnRH+	% of total GnRH+ co-labeled with virus
Olfactory Placode	9 (±2 SEM; n=8)	30% (± 7.5 SEM%; n=3)	10 % (±2.5 SEM%; n=4)
Neural Crest	10 (±3 SEM; n=17)	30%(±10 SEM%; n=17)	N/A

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Chapter 3

NEURAL CREST STEM CELLS FROM HUMAN EPIDERMIS OF AGED DONORS MAINTAIN THEIR MULTIPOTENCY IN VITRO AND IN VIVO

Adapted from Samaneh Moghadasi Boroujeni, **Alison Koontz**, Georgios Tseropoulos, Laura Kerosuo, Pihu Mehrotra, Vivek Bajpai, Surya Rajan Selvam, Pedro Lei, Marianne E Bronner, and Stelios Andreadis. (2019). "Neural crest stem cells from human epidermis of aged donors maintain their multipotency in vitro and in vivo". Scientific Methods 9, Article number: 9750. (https://www.nature.com/articles/s41598-019-46140-9)

ABSTRACT:

Neural crest (NC) cells are multipotent stem cells that arise from the embryonic ectoderm, delaminate from the neural tube in early vertebrate development and migrate throughout the developing embryo, where they differentiate into various cell lineages. Here we show that multipotent and functional NC cells can be derived by induction with a growth factor cocktail containing FGF2 and IGF1 from cultures of human inter-follicular keratinocytes (KC) isolated from elderly donors. They also maintained their multipotency, as evidenced by their ability to differentiate into all NC-specific lineages including neurons, Schwann cells, melanocytes, and smooth muscle cells (SMC). Notably, upon implantation into chick embryos, adult NC cells behaved similar to their embryonic counterparts, migrated along stereotypical pathways and contributed to multiple NC derivatives in ovo. These results suggest that KC-derived NC cells may provide an easily accessible, autologous source of

stem cells that can be used for treatment of neurodegenerative diseases or as a model system for studying disease pathophysiology and drug development.

INTRODUCTION:

The neural crest is a population of cells which arises during neurulation at the region between the neural plate border and the non-neural ectoderm. Upon formation of the neural tube, the neural crest cells are located at the dorsal margin of the neural tube. The neural crest cells then undergo an epithelial to mesenchymal transition to become migratory cells. They then migrate from the neural tube throughout the developing embryo to form a wide range of different derivatives.

Initiation of neural crest cell migration proceeds in a head-to-tail ward (rostrocaudal) wave, shortly following neural tube closure to form the central nervous system (CNS). After emigrating from the CNS, these cells move in a highly patterned fashion through neighboring tissues and localize in diverse sites. As a population, the neural crest is regionalized such that cells from different axial levels, designated cranial, vagal, trunk and lumbosacral follow distinct pathways (LeDouarin, 1982; Noden, 1975) and differentiate into different derivatives, with the cranial neural crest being the most complicated and diverse. Some cranial neural crest cells enter the branchial arches and form many of the cartilaginous elements of the facial skeleton. Others contribute to the ciliary ganglion of the eye and various cranial sensory ganglia. The cranial neural crest cells can be subdivided further into regions designated as caudal forebrain, midbrain, rostral hindbrain, and vagal/caudal

hindbrain neural crest cells; each group has a somewhat different pattern of migration and prospective derivatives. For example, neural crest cells originating in the midbrain migrate primarily as a broad, unsegmented sheet under the ectoderm; they contribute to derivatives ranging from the periocular skeleton, connective tissue and membranous bones of the face, to the ciliary and trigeminal ganglia (LeDouarin, 1982). Precise quail/chick grafting experiments have determined the regions of neural tube from which neural crest cells arise to contribute to cartilaginous elements. Cranial neural crest cells contribute to the quadrate, Meckel's cartilage and surrounding membrane bones, basihyoid cartilage in the tongue, and to membrane bones of the upper jaw and skull (Noden, 1978; Couly et al., 1992; 1993).

The tremendous diversity of derivatives formed by the cranial neural crest raises an intriguing question: can individual neural crest cells contribute to all derivatives of the face, or are precursors set aside that are already committed to particular lineages? The question of neural crest developmental potential has been best examined at trunk levels of the neural axis. Clonal analysis was first done in tissue culture, in which “clones” were derived from individual neural crest cells explanted under a variety of conditions (Sieber-Blum et al., 1981; Calloni et al., 2009). The results showed that some clones contributed to multiple cell types. Importantly, Stemple and Anderson further demonstrated that murine neural crest cells in culture were multipotent and had self renewal ability; moreover, different growth factors could bias clones toward particular fates (Stemple and Anderson, 1992).

Because cell culture can change cell behavior, it was important to also perform neural crest cell lineage in vivo. Analysis of the developmental potential of individual neural crest cells in vivo was first done for trunk neural crest cells by my pre-doctoral mentor (Bronner-Fraser and Fraser, 1988; 1989) using single cell injection of vital dyes in the dorsal neural tube. This was recently repeated in the mouse embryo using elegant Confetti technology (Baggiolini et al.,2015). Both methods show that many individual premigratory and migrating trunk neural crest cells are multipotent and able to form multiple derivatives. These studies reached identical conclusions despite using very different approaches in different model organisms.

Given that there are significant differences between cranial and trunk neural crest populations, the behavior of cranial and trunk neural crest clones may be very different. Even before migration, presumptive cranial and trunk neural crest cells exhibit differences. Premigratory cranial neural crest form an aggregate of rounded cells that appear to be segregated from dorsal neural tube cells (Theveneau et al., 2007) whereas premigratory trunk neural crest cells are columnar epithelial cells within the dorsal neural tube that are indistinguishable from other neuroepithelial cells (LeDouarin, 1982). Moreover, both in vivo transplantation and in vitro approaches have been used to compare the cell fate potentials of cranial and trunk neural crest. The results of these studies show that both have the ability to form melanocytes, glia, sensory neurons, and several kinds of autonomic neurons (Le Lievre et al., 1975; 1980; Lwigale et al. 2014; Simoes-Costa and Bronner, 2016). However, only cranial neural crest cells have the ability to develop into cartilage and bone, thus

demonstrating significant differences in developmental potential between cranial and trunk neural crest populations.

In vitro neural crest cells (NC) can be derived from embryonic stem cells as well as induced pluripotent stem cells (Lee et al., 2007; Bajpai et al., 2010; Mica et al., 2013*)*. However, similarly to NC cultures made from neural tube explants, these cell cultures have only a limited capability for self-renewal and had only a short time frame before they spontaneously differentiated, which made them difficult to use in a clinical setting.

A protocol developed in the Bronner lab found conditions that made long term maintenance of neural crest cells possible. Premigratory neural crest cells were collected from the neural ridges of chicken embryos and placed in a variety of cell culture conditions (Kerosuo et al., 2015). The effect of these different factors on readouts of classic neural crest genes such as FoxD3 and Sox10 showed that culture conditions were most optimal with bFGF, IGF1, and RA. These cells could be differentiated into neural crest cells *in vitro* and upon injection *in vivo* into the migratory neural crest stream of chicken embryos were found differentiated in canonical neural crest locations, as verified by immunoflorescent staining. What's more, these crestospheres maintained their self-renewal capabilities for several weeks, and the length of time was only limited by the duration of the experiment.

In addition to being cultured from embryonic cells, NC cells have also been isolated from various adult tissues including dorsal root ganglia, gut, heart, olfactory sheath, hair follicles

and craniofacial tissue (Sieber-Blum & Grim, 2004; Liu & Cheung, 2016; Achilleos & Trainor, 2012). These cells maintain their multipotency as they can be coaxed to differentiate into neuronal and glial cells, smooth muscle cells, melanocytes, bone cells, adipocytes, and chondrocytes.

Their huge range of derivatives and their ability to be cultured *in vitro* recommends these cells be used for applications including treatment for spinal cord injury, deafness, ocular repair or periodontal regeneration. However, clinical application is hampered by the need for genetic modification in reprogramming or the limited accessibility of adult tissues where they reside.

Recently, collaborators at SUNY Buffalo and former Bronner lab postdoctoral fellow Dr. Laura Kerosuo showed that NC cells can be derived from neonatal keratinocytes of the interfollicular epidermis, without introduction of transcription factors or reprogramming to pluripotency (Bajpai et al., 2017). However, it was not clear whether multipotent and functional NC cells can be derived from the adult epidermis of aged donors, who have the greatest need for cell therapies. Follow up experiments done by our collaborators were able to show that adult NC cells from elderly donors can also be obtained from epidermal cultures by treatment with a growth factor cocktail containing FGF2 and IGF1. Adult NC cells derived from KC cultures (KC-NC) from different donors expressed key NC markers including transcription factors SOX10, FOXD3, PAX3 and intermediate filament protein, NES. They also maintained their multipotency as evidenced by differentiation into all NC-

specific lineages including neurons, Schwann cells, melanocytes, and smooth muscle cells (SMC) (Figure X).

Here we show that lineage tracing experiments by implantation into chick embryos showed that KC-NC from aged donors could migrate along stereotypical pathways and differentiate into multiple NC derivatives in ovo, including neurons, glia, SMC and putative melanoblasts.

MATERIALS AND METHODS:

Isolation of epidermal cells

Skin from the right thigh of human cadavers ranging from 67 to 93 years of age was obtained from Gross Anatomy Lab of the University at Buffalo in accordance with appropriate guidelines and regulations. Written informed consent was provided before death by the donors who donated their bodies to the University at Buffalo for teaching purposes, scientific research, or such purposes as the University, or its authorized representatives, shall in their sole discretion deem advisable. The UB Institutional Review Board (IRB) determined that researchers using any materials from those donors do not need to get specific permission as UB already has blanket permission to use them as needed. The skin of the donors was harvested as it became available and the total number of donors used in this study was $n = 11$ (10 male and 1 female). After washing three times with phosphate-buffered saline (PBS), the skin tissues were dissected into small pieces (~1 cm × 1 cm) and enzymatically digested using dispase II protease (Sigma, St. Louis, MO) for 15–20 hr at 4 °C. The epidermis was separated from the dermis manually using fine forceps and then treated with trypsin-EDTA

(0.25%) (Life Technologies, Carlsbad, CA) for about 10–15 min at 37 °C. After filtering through a 70 μm cell strainer (BD Biosciences, Franklin Lakes, NJ), the cell suspension was centrifuged and resuspended in keratinocyte growth medium (KCM) containing 3:1 mixture of DMEM (high glucose) and Ham's F-12 medium (Life Technologies) supplemented with 10% (v/v) fetal bovine serum (FBS, Atlanta Biologicals, Flowery Branch, GA), 100 nM cholera toxin (Vibrio Cholerae, Type Inaba 569 B, Millipore, Burlington, MA), 5 μg/ml transferrin (Life Technologies), 0.4 μg/ml hydrocortisone (Sigma), 0.13 U/ml insulin (Sigma), $1.4 \times 10{-}4$ M adenine (Sigma), $2 \times 10{-}9$ M triiodo-L-thyronine (Sigma), 1x antibiotic-antimycotic (Life Technologies) and 10 ng/ml epidermal growth factor (EGF, added 3 days post-seeding, BD Biosciences). The resuspension was then cultured on a monolayer of growth arrested 3T3-J2 mouse fibroblast feeder cells. The harvested cells that were cultured in KCM formed colonies in 8 –10 days and the feeder cells were removed using versene treatment for about 10 min at 37 oC. The remaining cells were treated with trypsin-EDTA (0.25%); the trypsin was neutralized with PBS containing 10% FBS and the cells were cultured on collagen type I coated tissue culture plates (10 μg collagen type I per cm2; BD Biosciences) in keratinocyte serum free growth medium (KSFM, Epilife medium with Human Keratinocyte Growth Supplement, Life Technologies) until NC induction. KC were used immediately after isolation (passage 1) for all experiments. Neonatal cells were isolated from glabrous neonatal (1- to 3-day-old neonates) foreskin tissues that were obtained from the John R. Oishei Children's Hospital, Buffalo, NY according to IRB of John R. Oishei Children's Hospital. Samples were regularly discarded tissues from foreskin circumcisions. Since there was not any identifying data from patients, an exemption for obtaining patient

consent was granted by IRB of John R. Oishei Children's Hospital. All protocols were in accordance with appropriate guidelines and regulations.

Induction of neural crest stem cells

To obtain NC cells, KC were cultured at a density of 3–5 × 103 cells/cm2 on collagen I coated tissue culture plates and exposed to Neural Crest Induction Medium (NCIM) containing basal medium (EBM2 medium; Lonza, Basel, Switzerland) supplemented with 2% (v/v) FBS, 10 µg/ml heparin (Lonza), 100 µg/ml ascorbic acid (Lonza), 0.5 µg/ml hydrocortisone, 1x Gentamicin/Amphotericin-B (Lonza), 10 ng/ml fibroblast growth factor 2 (FGF2, Isokine, Iceland), and 10 ng/ml Insulin like growth factor 1 (IGF1, Lonza). After 2–3 days of induction, NC cells could be seen surrounding KC colonies and by day 10 they had proliferated extensively occupying almost the areas between KC colonies. At that time, NC cells were separated from KC by differential trypsinization for about 3 min and re-plated for further experiments. NC cells were derived from all donors (n = 11) and each assay as described below was conducted with cells from at least n = 3 donors.

Immunostaining and fluorescence microscopy

After washing with PBS, the cells were fixed with 4% (v/v) paraformaldehyde (10 min, room temperature (RT); Sigma), permeabilized using 0.1% (v/v) triton X-100 (Sigma) for 10 min at RT, washed 3 times with PBS and blocked with 0.01% (v/v) triton X-100 and 5% (v/v) normal goat serum (Life Technologies) in PBS. Then cells were incubated with primary antibodies overnight at 4 °C, followed by 1 hr incubation with secondary antibody (Alexa

488- or Alexa 594-conjuated anti-IgG antibody, Thermo Fisher Scientific, Grand Island, NY, 1:200 dilution) at RT and counterstained with Hoechst 33342 (Thermo Fisher Scientific) for 5 min at RT. Cells incubated with only secondary antibody served as negative controls. Images were taken using a Zeiss Axio Observer Z1 inverted microscope with an ORCA-ER CCD camera (Hamamatsu, Japan). The images were captured using fixed exposure time for each fluorescent dye for all samples. Fluorescence intensity and cells numbers were quantified using NIH ImageJ.

Differentiation of adult NC cells into NC derivatives

Schwann cell differentiation

Adult NC cells were plated on poly-L-ornithine/laminin coated plates and cultured in Schwann cell (SC) differentiation medium containing EBM2 as basal medium, 2% (v/v) FBS, 100 ng/ml ciliary neurotrophic factor (Life Technologies), 100 ng/ml NRG1, 4 ng/ml FGF2, 200 mg/ml ascorbic acid, 0.5× Glutamax (ThermoFisher Scientific), and 10 μM SB431542 (Sigma) for 5 weeks.

Melanocyte differentiation

Adult NC cells were cultured in EBM2 basal medium supplemented with 5% FBS, SCF (100 ng/ml), endothelin-3 (200 nM), WNT1 (50 ng/ml), FGF2 (10 ng/ml), insulin (5 μg/ml), cholera toxin (1 pM), 12-O-tetra-decanoylphorbol-13-acetate (TPA, 10 nM; Sigma) and SB431542 (10 μM) for five weeks. At that time, we examined melanin secretion, using the L-DOPA assay, for which adult NC-Mel were fixed with 4% (w/v) paraformaldehyde for

20 min at room temperature. After washing three times with PBS, the cells were incubated with freshly prepared 5 mM L-DOPA (Sigma) overnight at 37 °C, fixed with 4% (v/v) paraformaldehyde for 20 min at RT, washed with PBS, and visualized using bright field microscopy.

Smooth muscle cell (SMC) differentiation

KC-NC were induced to SMC in DMEM plus 10% (v/v) FBS and 10 ng/ml TGF-β1 for two weeks. ASMC were used as the positive control.

Peripheral neuron differentiation

Adult NC cells were cultured on poly-L ornithine (100 ng/ml; Sigma)/laminin (10 mg/ml; EMD Millipore, Billerica, MA) coated dishes and exposed to neuron differentiation media containing Neurobasal plus medium (Thermo Fisher Scientific) with BMP2 (10 ng/ml; R&D systems, Minneapolis, MN), SB431542 (10 μM), B27 plus (Thermo Fisher Scientific), N2 supplement (R&D systems), Brain-derived neurotrophic factor (BDNF; 10 ng/ml; Thermo Fisher Scientific), Glial cell-derived neurotrophic factor (GDNF;10 ng/ml; Sigma), Nerve growth factor (NGF; 10 ng/ml; R & D systems), Neurotrophin 3 (NT3; 10 ng/ml; Sigma), ascorbic acid (200 μM; Sigma) and cyclic adenosine monophosphate (0.5 mM cAMP; Sigma), CHIR 99021 (0.5 μM, only on day 1; Sigma), 2% FBS (from day 1–5), IWP-4 (100 nM days 4–6; 1 μM thereafter; Tocris Bioscience, Minneapolis, MN).

In ovo transplantation of KC-NC

Adult KC- NC stem cells were transduced with lentivirus containing CMV promoter driving expression of the ZsGreen+ reporter. About 50–60% of cells were ZsGreen+ as evidenced by fluorescence microscopy. KC-NC or control KC were dissociated using 2 mL of Accuprime (#AM-105, Innovative Cell Technologies Inc., San Diego, CA) and incubated at 37 °C for 5 minutes. The cells were washed twice with 1 mL of Ringer's balanced salt solution, and spun down for 7 minutes at 200 G, resuspended into 10 to 20 μL of cell medium, and loaded into a thin pulled glass needle pipette. The cells were injected into the migratory cranial NC stream of Hamburger-Hamilton Stage 9–12 chick embryos. In total, 157 embryos were successfully injected with experimentally induced NC cells, and 55 with control cells (undifferentiated keratinocytes). Embryos were examined for visible GFP fluorescence under a Leica fluorescent microscope to determine the efficiency of injections, covered with sterile surgical tape, and incubated at 37 °C. After 48–72 hours, the surviving embryos were dissected out, fixed with 4% paraformaldehyde in PBS overnight at 4 °C, and washed 3 times with PBS. Thirty-nine experimental embryos (25% survival rate) and 19 control embryos (35% survival rate) survived and were processed.

Fixed embryos were embedded in gelatin and sectioned transversely at 14 μm on a cryostat. Sections were examined under a fluorescent Apotome microscope (Zeiss Axioscope 2 and Zeiss ApoTome.2) for GFP signal. Sections containing GFP positive cells were blocked with a 2.5% goat and 2.5% donkey serum solution in PBS-Tween 0.2%, and antibodies were added to the same blocking solution. Immunostaining was performed with the following antibodies: for glia, BLBP (ABN14, EMD Millipore, 1:200, antigen retrieval was performed

by placing slides in sodium citrate buffer, pH 6, in a 68 °C water bath overnight, prior to blocking); for neurons HuC/D (Invitrogen/molecular probes 16A11 1:100); for smooth muscle, αSMA (Sigma A5228 1:2000); for nuclei, DAPI (1:1000). Secondary Alexa dye-conjugated antibodies (Molecular Probes) were used at 1:1000. Slides were imaged using fluorescence microscopy (Zeiss Axioscope 2 and Zeiss ApoTome.2).

In ovo transplantation of KC-Schwann Cells

KC-Schwann Cell or control KC-NC were dissociated using 2 mL of Accuprime (#AM-105, Innovative Cell Technologies Inc., San Diego, CA) and incubated at 37 °C for 5 minutes. The cells were washed twice with 1 mL of Ringer's balanced salt solution, and spun down for 7 minutes at 200 G, resuspended into 10 to 20 μL of cell medium, and loaded into a thin pulled glass needle pipette. The cells were injected into the migratory cranial NC stream of Hamburger-Hamilton Stage 10–12 chick embryos. In total, 52 embryos were successfully injected with experimentally induced NC cells, and 33 with control cells (undifferentiated keratinocytes). Embryos were covered with sterile surgical tape, and incubated at 37 °C. After 48–72 hours, the surviving embryos were dissected out, fixed with 4% paraformaldehyde in PBS for 30 minutes at room temperature, and washed 3 times with PBS. 6 experimental embryos (12% survival rate) and 5 control embryos (15% survival rate) survived and were processed.

Fixed embryos were embedded in gelatin and sectioned transversely at 14 μm on a cryostat. Sections were examined under a fluorescent Apotome microscope (Zeiss Axioscope 2 and

Zeiss ApoTome.2) for GFP signal. Sections containing Hu nuclei positive cells were blocked with a 2.5% goat and 2.5% donkey serum solution in PBS-Tween 0.2%, and antibodies were added to the same blocking solution. Immunostaining was performed with the following antibodies: for glia, BLBP (ABN14, EMD Millipore, 1:200, antigen retrieval was performed by placing slides in sodium citrate buffer, pH 6, in a 68 °C water bath overnight, prior to blocking); for neurons HuC/D (Invitrogen/molecular probes 16A11 1:100); for smooth muscle, αSMA (Sigma A5228 1:2000); for nuclei, DAPI (1:1000). Secondary Alexa dye-conjugated antibodies (Molecular Probes) were used at 1:1000. Slides were imaged using fluorescence microscopy (Zeiss Axioscope 2 and Zeiss ApoTome.2).

RESULTS:

Adult neural crest stem cells derived from keratinocyte cultures

Previously we showed that neural crest stem (NC) cells can be isolated from the interfollicular epidermis of glabrous skin from 1–3 day old neonates. However, it was not clear that NC-like cells can also be derived from adult epidermis. To this end, we derived NC cells from epidermal KC of human skin tissues of adult donors ranging from 67 to 93 years of age (n = 11 donors). KC were initially cultured in calcium free medium (KSFM). When the medium was changed to the NC induction medium (NCIM consisted of EBM2 basal medium containing FGF2, IGF1, ascorbic acid, hydrocortisone, heparin, and 2% FBS), KC formed colonies that were surrounded by a number of small, spindle shaped cells 5–6 days later. Immunostaining showed that these cells expressed key epidermal NC markers

including lineage-specific transcription factors such as SOX10, FOXD3, PAX3, the NGF receptor (NGFR) and the intermediate filament protein, NES (Fig. 1A). Almost all cells expressed NES; the vast majority expressed Pax3 ($92.68 \pm 6.75\%$), FoxD3 ($97.3 \pm 0.99\%$), and NGFR ($87.7 \pm 4.01\%$), while about $40.0 \pm 2.96\%$ of cells were positive for Sox10 after 14 days in NCIM (4 fields of view containing $n \geq 500$ cells) (Fig. 1B).

Differentiation of adult NC cells to functional neural crest derivatives

To address this hypothesis, we examined the propensity of adult KC-NC to differentiate into NC derivatives, including Schwann cells, neurons, melanocytes, and smooth muscle cells.

Schwann cells

NC cells differentiated into Schwann cells in the presence of differentiation medium containing EBM2 basal medium supplemented with 2% FBS, 100 ng/ml CNTF, 100 ng/ml NRG1, 4 ng/ml FGF2, 200 μg/ml ascorbic acid and 0.5x Glutamax, for 5 weeks. Immunostaining showed that almost all the cells were positive for S100B, PLP1, and MPZ (Fig. 3A).

Peripheral neurons

NC cells differentiated into peripheral neurons using neuron differentiation medium (Neurobasal plus media with BMP2 (10 ng/ml), SB431542 (10 μM), B27 plus, N2 supplement, Brain-derived neurotrophic factor (BDNF; 10 ng/ml), Glial cell-derived neurotrophic factor (GDNF; 10 ng/ml), Nerve growth factor (NGF; 10 ng/ml), Neurotrophin

3 (NT3; 10 ng/ml), Ascorbic acid (200 μM) and cyclic adenosine monophosphate (0.5 mM cAMP), CHIR 99021 (0.5 μM, only on day 1), 2% FBS (from day 1–5), IWP4 (100 nM days 4–6; 1 μM thereafter). After 14 days in differentiation medium, the cells developed long processes and expressed typical neuronal markers such as Peripherin (83.85 ± 0.3%, n = 567 cells) and TUBB3 (59.48 ± 0.2%, n = 1,832 cells) (Fig. 3B,C).

Melanocytes

For melanocyte differentiation, NC cells were cultured in EBM2 basal medium supplemented with 5% FBS, SCF (100 ng/ml), endothelin-3 (200 nM), WNT1 (50 ng/ml), FGF2 (10 ng/ml), insulin (5μg/ml), cholera toxin (1 pM), 12-O-tetra-decanoylphorbol-13-acetate (TPA, 10 nM) and SB431542 (10 μM). After 5 weeks of differentiation, 52 ± 6.33% of the cells expressed the melanocyte-specific transcription factor, MITF and 40 ± 6.61% expressed the pre-melanosome transmembrane glycoprotein, PMEL (n = 780 cells) (Fig. 3D). Notably, NC-derived melanocytes produced melanin, clearly indicating tyrosinase activity–specific for melanocytes, as evidenced by the L-DOPA assay (Fig. 3E).

Smooth muscle cells

NC cells were coaxed to differentiate into SMC in DMEM supplemented with 10μg/ml TGF-β1 for two weeks. Almost all of the NC-SMC showed positive staining for ACTA2, CALD1, and MYH11, and about 92.85 ± 8.5% (n = 308 cells) expressed CNN1 (Fig. 4A).

NC cells migrate and differentiate into NC lineages in ovo

We performed lineage tracing experiments to examine the ability of KC-NC to migrate towards stereotypical pathways in vivo. To this end, ZsGreen labeled KC or KC-NC that were transplanted into the head mesenchyme of 8–13 somite host chick embryos (Fig. 5B) were analyzed either 48 hours (n = 16) or 72 hours (n = 23) post-transplantation.

The results showed that KC-NC were predominantly detected in locations populated by neural crest-derived cells (Fig. 5A). The majority, 60% of the KC-NC (227 cells out of 382; n = 8) localized to cranial ganglia, significantly different from only 7% of the control KC (18 cells out of 253; n = 6, p = 0.002). Similarly, 14% of the KC-NC (54 cells out of 382; n = 8) were located in the branchial arches as compared to 3% of KC controls (7 out of 253; n = 6), although the results were not statistically significant (p = 0.59).

The KC-NC contributed to the full repertoire of NC derivatives, from HuC/D expressing neural (Fig. 5D) and BLBP positive glial cells (Fig. 5F), to mesenchymal αSMA positive smooth muscle lining the blood vessel walls (Fig. 5E). KC-NC also gave rise to presumptive melanoblasts below the ectoderm, although the time point for the analysis was too early to detect the melanocyte lineage marker MitF (Fig. 5C).

In contrast, 75% (189 cells out of 253; n = 6 embryos) of control KC were preferentially found in the mesenchyme, corresponding to the original site of injection, whereas only 14% (52 cells out of 382; n = 8) of KC-NClocalized in the mesenchyme (p = 0.0013).

These results show that KC-NC behave similarly to embryonic NC cells in ovo and can contribute to multiple NC derivatives, providing strong support of NC phenotype.

DISCUSSION:

It was previously found that neural crest (NC) stem cells could be derived from neonatal human epidermal keratinocytes (KC) without genetic introduction of transcription factors or reprogramming to the pluripotent state. However, it was not clear whether NC cells could also be derived from the skin of adult donors, who are most likely in need of cellular therapies.

Collaborators at SUNY Buffalo reported for the first time that NC cells can be obtained from the epidermis of older adult donors ranging from 67 to 93 years of age. Adult NC cells derived from KC cultures expressed key NC markers including lineage-specific transcription factors such as SOX10, FOXD3, PAX3, the intermediate filament protein, NES and cell surface receptor, NGFR (p75NTR), similar to that observed with neonatal KC-NC.

Just as with neonatal NC cells, adult KC-NC could be coaxed to differentiate into functional neurons, Schwann cells, melanocytes and SMC, in vitro. Most notably, upon transplantation into chick embryos, KC-NC migrated along stereotypical pathways and gave rise to multiple NC derivatives, including neurons, glial cells, SMC lining the vascular wall, and presumptive melanoblasts in the skin. These lineage tracing experiments in chick embryos provide strong support of the phenotype and multipotency of KC-NC.

Since these cells can be derived from the human epidermis with no genetic modification or reprogramming to the pluripotent state, they have the potential to be used for treatment of neurodegenerative diseases—for which cell source remains a significant hurdle—as well as for modeling human diseases of the central or peripheral nervous system ,e.g., neurocristopathies. Therefore, this readily accessible source of NC cells may have significant impact on regenerative medicine as well as understanding human disease and facilitating drug discovery.

We showed that adult NC cells can be derived from human interfollicular KC from older donors without direct reprogramming or reprogramming to pluripotency. These cells maintained their multipotency in vitro and in vivo. Given the accessibility of human epidermis and ease of isolation, adult KC-NC have great potential for use in stem cell therapy, disease modeling, and drug discovery for treatment of neurodegenerative disorders.

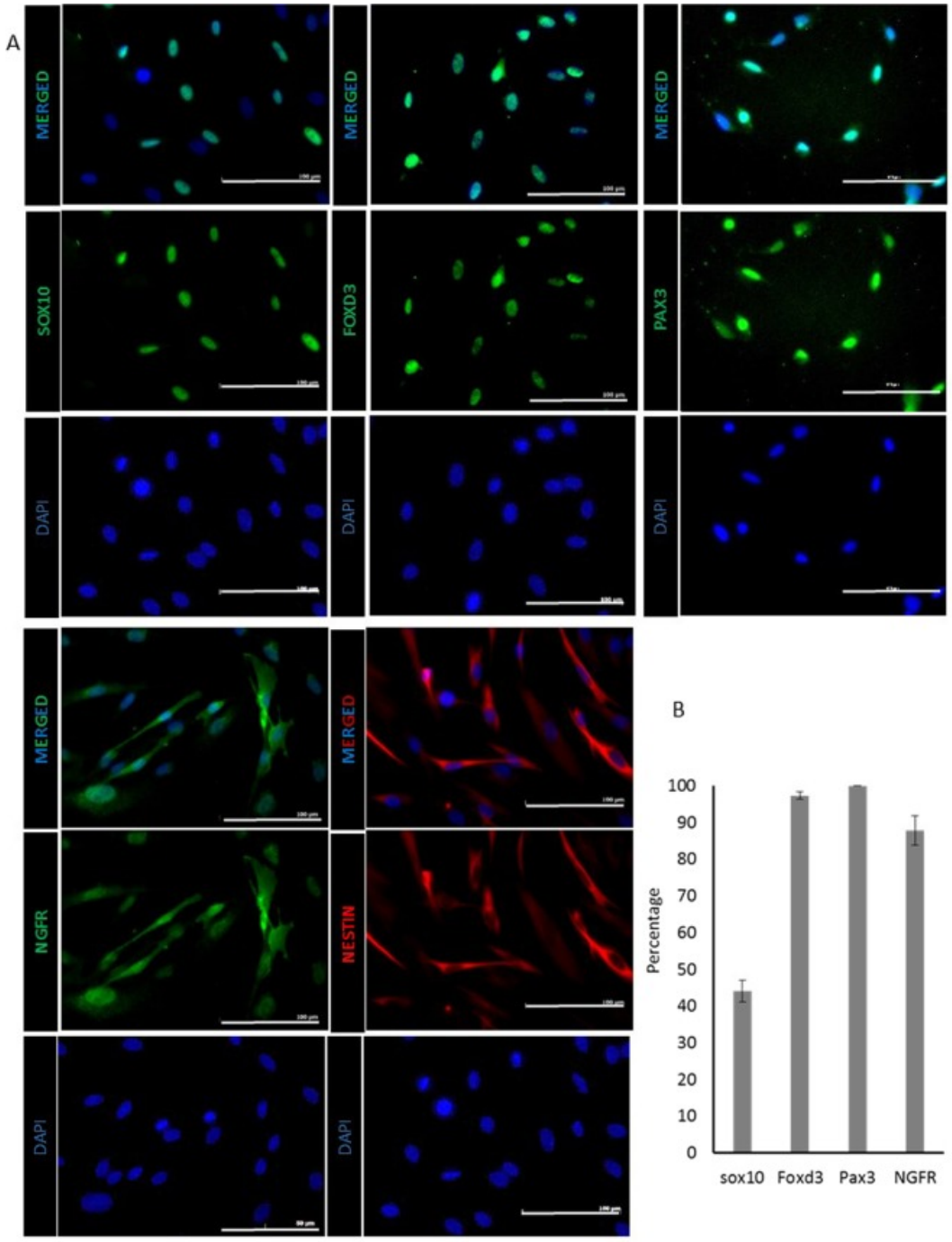

Figure 3.1. Adult NC cells derived from keratinocyte cultures express NC specific markers. (A) Immunostaining of adult NC cells for SOX10, FOXD3, PAX3, NGFR and NESTIN. Scale bar is 100 μM. (B) Percentage of adult NC cells expressing SOX10, FOXD3, PAX3, and NGFR after two weeks of culture. All values are mean ± SD. Each experiment was repeated three times.

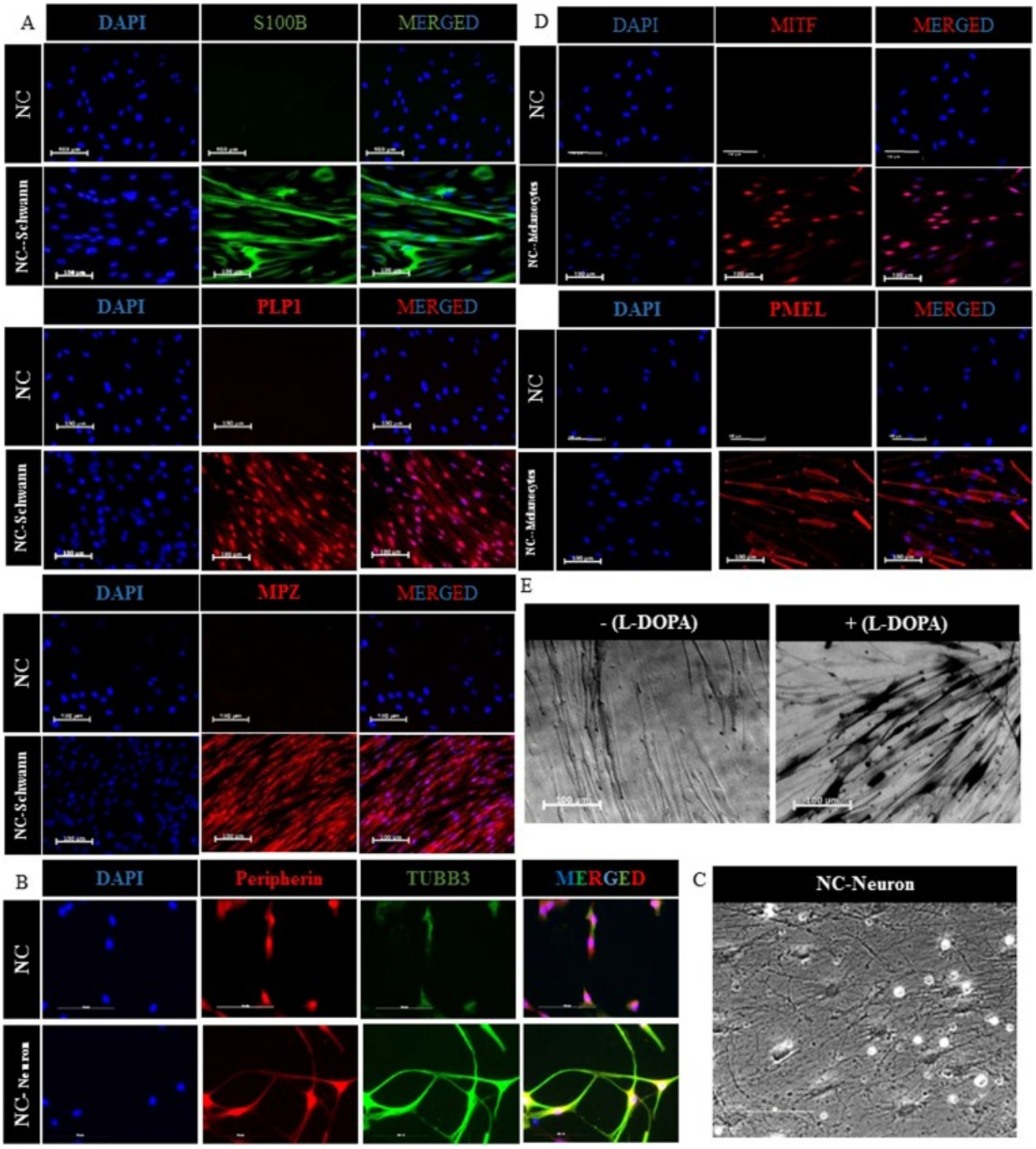

Figure 3.2. Differentiation of adult KC-NC to functional neural crest derivatives. Immunostaining for (**A**)Schwann cell specific markers including. S100B, PLP1, and MPZ, and **(B)** peripheral neuron specific markers, Peripherin and TUBB3. **(C)** Phase image shows the morphology of adult NC-derived neurons. **(D)** Melanocyte specific markers MITF and PMEL. Scale bar is 100 µm. **(E)** L-DOPA assay showing melanin secretion indicating tyrosinase activity.

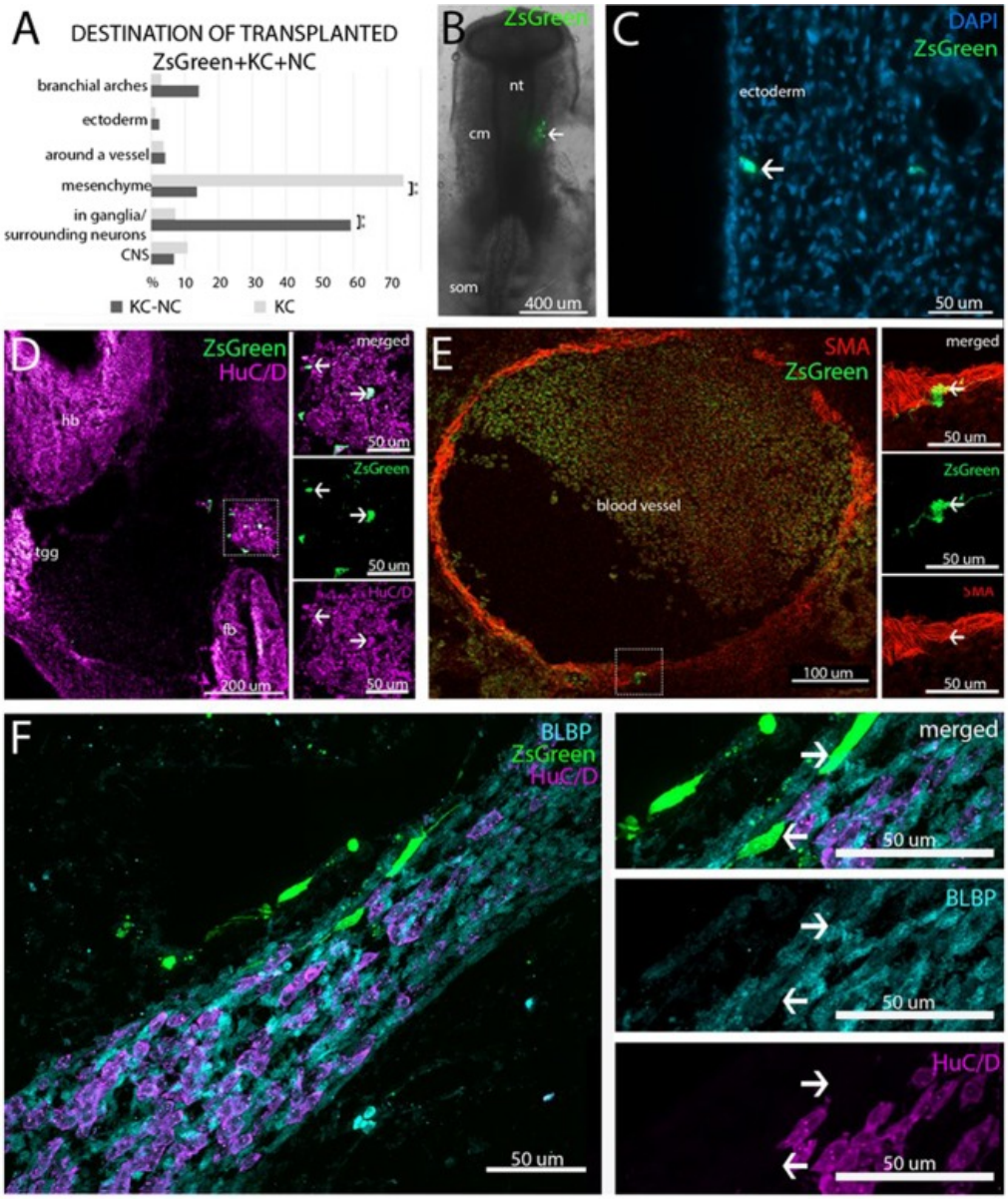

Figure 3.3. Adult KC-NC migrate to neural crest sites and differentiate into appropriate derivatives in ovo. **(A)** Summary of locations in which ZsGreen positive transplanted cells were found in 3–4-day-old chicken embryos in representative embryos. Percentage of experimental transplanted cells detected in each target structure in the developing chick embryos (n = 8 embryos; total number of detected ZsGreen+ cells = 382 out of ~2000 transplanted cells) compared with the percentage of control keratinocytes (n = 6 embryos; total number of detected ZsGreen+ cells = 253 out of ~3000 transplanted cells). **(B)** An image showing transplanted ZsGreen+ KC-NC in the cranial mesenchyme (cm) of a 8–13 somite (som) host chick embryo immediately after injection; neural tube = nt; cm = cranial mesenchyme; som = somite. **(C)** Putative ZsGreen+ melanocytes 72 hours post injection underneath the cranial ectoderm. **(D)** HuC/D and ZsGreen double-positive neurons within the trigeminal ganglion (tgg); fb = forebrain; hb = hindbrain. **(E)** A SMA+ cranial blood vessel with a ZsGreen/SMA double positive transplanted cell. **(F)** ZsGreen/BLBP double positive glial cells (presumably Schwann cells) localized in a HuC/D-positive nerve bundle.

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Chapter 4

FUTURE DIRECTIONS

PROPOSAL: GnRH Neurons Across the Tree of Life

Reproductive success is a huge part of an organism's fitness, and is integral to the evolution of species (Darwin, 1859). It follows, then, that any biological system which plays a large role in the reproductive fitness of an organism has an important role in evolution as a whole.

The GnRH (gonadotropin releasing hormone) system is perhaps the most important endocrine system within vertebrates. GnRH neurons are responsible for the production of gonadotrophin hormone, which are released into the pituitary system and travel through the blood stream to the gonads (Marques et al., 2000). GnRH neurons are present early in embryogenesis, but increase their secretion of gonadotropins at the beginning of adolescence, triggering the onset of puberty, and are critical for reproduction in adulthood (Cariboni et al., 2007; Cho et al., 2019). In fact, mutations causing malfunction in GnRH neurons or their migration from OE to hypothalamus result in reproductive disorders such as Kallman's syndrome and infertility (Cadman et al., 2007).

GnRH neurons first make an entrance in the olfactory epithelium (OE) early in development, then migrate out of the OE and travel through the nasal septum into the hypothalamus, where they remain through adulthood (Schwanzel-Fukuda, 1989).

There are three isoforms of GnRH found across jawed vertebrates, with most species containing more than one, or even all three variants (Lethimonier et al., 2003). These different variants are thought to arise from the two whole genome duplications that occurred early in vertebrate evolution (Kim et al., 2012). *GnRH1* is the major player in hypothalamic hormone regulation, while the function of the other two isoforms, *GnRH2* and *GnRH3* are likely supportive endocrine roles (Whitlock et al., 2019). In zebrafish, *GnRH1* is expressed on center stage in the hypothalamus, whereas *GnRH2* is expressed preferentially in the midbrain and *GnRH3* is found within the olfactory nerve (Cho, 2018).

Different phyla possess different variants of GnRH genes. For instance, it was long thought that *GnRH3* was exclusive to only teleost fish; however, more recent studies have shown that many other species express *GnRH3*, although it remains to be seen in tetrapods (Okubo & Nagahama, 2008). *GnRH3* and other GnRH variants have also been found within lamprey, a model for jawless vertebrates (Gaillard et al., 2018). What's more, pre- GnRH like molecules have also been found in older lineages such as amphioxus, tunicates, and even within invertebrates like *Drosophila* (Roch et al., 2010). The wide presence of GnRH molecules across the tree of life presents the intriguing notion that these neurons perhaps even precluded the entire pituitary system itself.

Although these GnRH neurons are of great importance to the survival and reproduction of animals across all taxa, relatively little is known about these neurons in non-model systems. Most of the knowledge we have of the GnRH system comes from mammals and teleost fish. This is an unfortunate oversight, as investigation of multiple

vertebrate taxa's GnRH structure would be useful in potentially elucidating the evolution of these neurons throughout vertebrates. As a neuron that has direct effects on the success of organism reproduction, these investigations are directly related to organismal success and species evolution.

GnRH Across Taxa

Cartilaginous fish are understudied with regards to the GnRH system. Cartilaginous fish are some of the oldest extant vertebrate taxa on Earth and are the closest lineage to most teleost fish (Gaillard et al., 2008). They are one of the most successful clades given their wide distribution across oceans and time and are among the top predators in the ocean for which scent is a key input in the aqueous milieu they live (Hoover, 2010). Their unique phylogenetic position presents an opportunity to investigate a more ancestral form of the GnRH system, which could give insight into the evolution of this system as a whole.

Until recently, only a handful of cartilaginous fish had been looked at for GnRH neuron presence; the elephant shark, the ratfish and the spiny dogfish were all shown to all have *GnRH2* (Lovejoy et al., 2017). A more recent study in 2018 showed that three representatives of cartilaginous fish, the cat shark, the whale shark, and the elephant shark, actually possess all 3 variants of GnRH and that these variants are expressed in different regions of the brain (Gaillard et al., 2018). However, so far there has only been

one study that illustrates the expression patterns of these variants within actual tissue samples. This study, done on the bonnethead shark, showed that *GnRH1* and *GnRH2* were both localized in the olfactory nerve, which contrasts to what is seen in studied teleosts and mammals where *GnRH3* is expressed in the olfactory nerve and *GnRH1* and *GnRH2* are respectively found in the hypothalamus and midbrain (Moeller & Meredith, 2010).

Outside of the chicken, the GnRH system of reptiles is also understudied. At present, GnRH neurons and receptors have been characterized in a few reptiles such as alligators, skinks, the Italian wall lizard, the tegu, and the leopard gecko (Powell et al., 1986; Ikemoto & Park, 2007; Sherwood & Whittier, 1988). Another experiment showed that mammalian and chicken GnRH forms could stimulate GnRH release *in vivo* for snakes, as well as for the turtle and the amphibious frog (Licht et al., 1987). Again, these studies focused only on characterizing GnRH from brain extracts rather than intact tissue samples, and none of them examined the expression patterns of multiple GnRH variants *in vivo*.

I propose to conduct a closer examination of the variants of GnRH neurons across developmental time in various representative taxa of cartilaginous fish and reptiles. Furthermore, I intend to identify and experimentally confirm a molecular regulatory region for GnRH2, the most highly conserved variant across vertebrates, within the chicken embryo. Once this regulatory region is identified, the sequence can also be used to probe the genomes of other non-model taxa. Finally, I would like to perform lineage

analysis using DiI in a non-model system to probe the embryonic origins (neural crest vs. placode) of the GnRH neurons in more ancient taxa.

Specific Aim 1: Determine the presence and localization of each GnRH variants in the genomes of multiple representatives of cartilaginous fish and reptiles through genome search and in situ hybridization of histological samples from representative taxa.

To this end, we have samples on hand of late stage embryos of the brownbanded bamboo shark *Chiloscylium punctatum* (Mueller and Henle, 1838), representatives of the skate genus *Leucoraja* (Malm, 1877), the common chicken *Gallus* (Linneaus, 1758), the tokay gecko, *Gekko gecko* (Linneaus, 1758), the American crocodile *Crocodylus acutus* (Cuvier, 1807), chameleon, and the painted turtle, *Chrysemys picta* (Schneider, 1783).

As a first step, we will probe the genomes of these six different taxa for the presence of the three GnRH variants with known GnRH nucleotide sequences from the most closely related taxa.

In a proof of concept experiment, a NCBI BLASTP search of the *C. punctatum* genome using the pre-GnRH2 protein sequence from the whale shark *Rhincodon typus* (Smith, 1828) yielded a hit for a hypothetical protein with over 90% identity with the query pre-GnRH sequence. This particular hypothetical protein was confirmed as a putative GnRH2

gene by the presence of the highly conserved decapeptide sequence, characteristic of the GnRH2 protein family in the chicken, which encodes the GnRH hormone itself (**Figure 4.1**).

With knowledge of not only the presence of GnRH variants but also the protein and nucleotide sequence and location in the genome, we can create cDNA species-specific sequences for each of the GnRH variants, followed by *in situ* probes. With these probes in hand, we can then investigate the expression of these various GnRH variants in actual histological tissue samples of these organisms.

This study will provide more data on the localization of these different variants across the brain. As of now, the specific functions of *GnRH2* and *GnRH3* are only postulated and not known for certain. Having more knowledge of the brain regions that express GnRH variants in more ancient lineages could give further insight into the conservation of these variants within regions of the brain and inform predictions as to their functions.

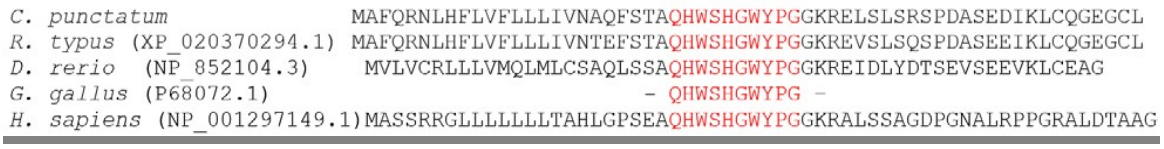

Figure 4.1: Protein sequence alignment (with accession numbers) shows highly conserved decapeptide sequence is found within *C. punctatum*, a previously unprobed taxa.

Potential Pitfalls

The main drawback of this aim is its reliance on already published genomes and genome annotations for lesser studied organisms. It may be found that a particular organism proposed for our study does not have a suitable published genome to probe, or that there are no characterized GnRH variants in closely related taxa to use as a query. In a case like this, the genome of the *most closely related taxa with a suitable genome* will be probed with an appropriate query taxon.

We can also try using already available commercial antibodies against the GnRH peptide. The GnRH hormone is composed of a highly conserved ten amino acid length region that is incredibly resistant to mutation. Immunohistochemistry markers for the GnRH peptide may work for multiple taxa, especially for GnRH2 which overall sequence is highly conserved.

Specific Aim 2: Investigate the molecular regulators (i.e. enhancers, promotors) that govern GnRH expression through genome search and genome perturbation experiments in chick embryos.

I propose to investigate the presence of GnRH1 enhancer and promoter regions within the genome of the chicken. Chickens are a member of the reptilian lineage, and therefore are more closely related to the other taxa of our studies than mice or other mammals. The chicken embryo is a great model for early and late stage embryonic perturbation studies,

and is robust and amenable to perturbation. The chicken embryo also develops outside of the mother, which makes in vivo embryonic experiments and collection even easier.

We will begin by probing the genome in chicken for an enhancer sequence with high sequence identity to the already publicized GnRH1 enhancer from cell lines (Whyte et al., 1995). These will be further confirmed by confirming the presence of binding sites for already known partners such as Dlx and GATA (Iyer et al., 2010). Once potential regulatory sequences have been found, they will first be tested by truncation analysis to determine the critical sequence involved in regulation. Due to the high throughput this experiment will require, initial truncation analysis will be done through in ovo electroporations of experimental plasmids at stage HH10 when GnRH is already present in the pre-placodal region, and the investigation on the effect on GnRH transcript will occur using in situ or immunostaining analysis at HH12, when the olfactory placode is clearly specified (Bhattacharyya & Bronner-Fraser, 2008). Once the critical regulatory region or regions are identified, they can be tested in vivo.

Using the chicken embryo, we will also perform in vivo experiments for the effect of perturbations in the found enhancer of GnRH1 on GnRH neuron migration during development. First, we will introduce an expression vector driving the GnRH1 enhancer into the embryo at various time points to determine when enhancer expression begins. Once this is done, we will utilize a CRISPR/Cas9 electroporation protocol that was recently developed in the chicken embryo to perform GnRH2 regulatory region knockouts to investigate effects on GnRH migration phenotype (Ghandi et al., 2017). For example,

we could choose to investigate how deletion of the binding region for Dlx effects the subsequent GnRH migration.

We will then perform thorough genome searches for the GnRH1 regulatory region across notable taxa from cartilaginous fish to vertebrates using the chicken sequence from Aim 2 as a query. Once found, in situ hybridization experiments with species-specific probes will illuminate the spatial expression of this important regulatory region throughout development and may give insight into the evolution of this regulatory mechanism across vertebrates.

The functional studies of this aim rely on 1) the presence of an enhancer region with high sequence similarity in chickens, and 2) that this enhancer is critical for GnRH differentiation during early development. In particular, the last condition could prove to be false. There may be multiple mechanisms that control GnRH fate besides one particular enhancer, and therefore functional overexpression or knock out through electroporation could have little or no effect on GnRH differentiation and developmental migration because of redundancy and compensation. In this case, instead of focusing on the enhancer itself, I would focus on the effects of overexpressing or knocking out known transcription factors that bind with the enhancer, and investigate how this changes GnRH neuron development, which can be readily done.

Potential Pitfalls

The functional studies of this aim rely on 1) the presence of an enhancer region with high sequence similarity in chicken, and 2) that this enhancer is critical for GnRH differentiation during early development. In particular, the last condition could prove to be false. There may be multiple mechanisms that control GnRH fate besides one particular enhancer, and therefore functional overexpression or knock out through electroporation could have little or no effect on GnRH differentiation and developmental migration because of redundancy and compensation. In this case, instead of focusing on the enhancer itself, I would focus on the effects of overexpressing or knocking out known transcription factors that bind with the enhancer, and investigate how this changes GnRH neuron development, which can be readily done.

Specific Aim 3: Characterize GnRH neuron origin and migration in a non-model organism by using DiI in turtle embryos to label early olfactory placode cells and trace through development.

Among model organisms, there has been a substantial amount of controversy regarding the embryonic origin of GnRH neurons. GnRH neurons were originally described as originating from the olfactory placode, but it was later proposed that a subset of GnRH neurons originate from the cranial neural crest population (Wray et al., 1989; Whitlock et al., 2003; Yamamoto et al., 1996). Since then, some studies report a dual origin of GnRH cells, with a portion of neurons originating in the placode and another portion originating from the neural crest (Forni et al., 2011). Still other studies have reported a uniquely placodal origin for the GnRH neurons (Sabado et al., 2011).

Characterizing the GnRH neuron development of a novel organism such as the turtle could add support to the ancient origins of the GnRH cells and would provide more insight into the more basal properties of this system in the tree of life.

I propose to use DiI injection to label the olfactory placode of turtle embryos following commitment to the olfactory placode fate. In chickens, commitment to the olfactory fate occurs at HH18, when the olfactory pit is fully formed and begins to ingress to form the olfactory epithelium (Bhattacharyya & Bronner-Fraser, 2008). Through injecting a lipophilic dye into cells of the newly formed olfactory pit, prior to this ingression, we can trace these placodal cells through development of the olfactory epithelium and migration of GnRH neurons to the forebrain. This will confirm whether the olfactory placode gives rise to GnRH neurons in the turtle. DiI in combination with our previously made in situ probes and immunohistochemistry antibodies from Aim 1 and Aim 2 will allow us to characterize the origins and development of the GnRH neurons within a novel organism.

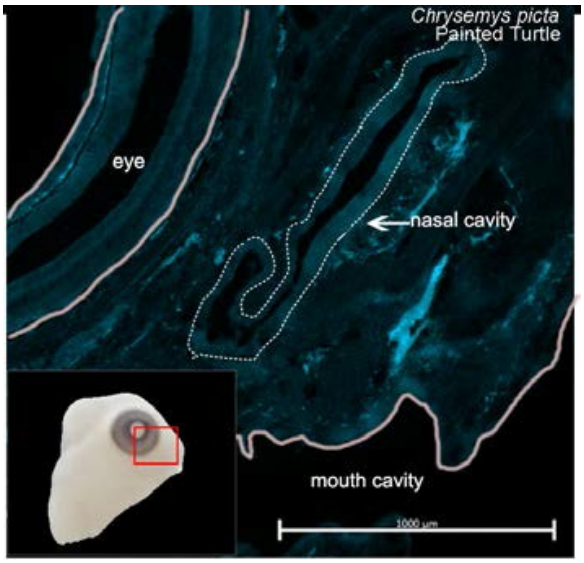

Figure 4.2 : A sagittal histology section in the cranial region of *Chrysemys picta* (denoted by red box in whole embryo image). Note the large olfactory cavity!

Turtles are an ideal novel organism for studying the olfactory system. Their embryonic development has already been mapped out into distinct developmental stages (Jefferey et

al., 2017). DiI experiments have also already been successfully performed in them by my sponsoring scientist (Goldberg et al., 2020). Due to their phylogenetic position, their development is also remarkably similar to that of the chicken which has characterized thoroughly both the molecular dynamics and morphological development of olfactory placode specification and commitment [Bhattacharyya et al., 2004; Bhattacharyya & Bronner-Fraser, 2008). What's more, turtles have the added advantage of having incredibly large olfactory systems, which would ease investigation as well (**Figure 4.2**) (Saito et al., 2000)!

Pitfalls

One drawback of DiI is the fact that it is only viable as a method of visualization for up to about three days post-injection due to the dye diluting with every cellular division. This should not effect our study, as ingression and identification of early GnRH neurons occurs well within this time frame in the chicken, which has GnRH neurons visible as early as Day 6 of development, 3 days following the commitment of the olfactory pit. If, however, this is a problem, there are alternative methods that can be used to label olfactory placode cells. For example, introduction of a retrovirus which encodes a fluorophore gene can be used to permanently label the olfactory pit cells which can then be traced as well. Although this method works well, it is also incredibly labor intensive and expensive, and therefore should only be considered as a back-up.

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