Abstract

Title of Document:	THE ROLE OF THE ADHERENS JUNCTION PROTEIN αN-CATENIN IN NEURAL CREST-DERIVED TRIGEMINAL GANGLIA FORMATION
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Abstract:

Neural crest cells (NCCs), a transient, multipotent population of cells that arise during neurulation, are a class of cells crucial to normal vertebrate development. NCCs must be tightly regulated by molecular and structural cues to de-adhere from the neural tube, migrate to their final destinations in the developing embryo, and differentiate to contribute to a variety of structures throughout the adult body. αN-catenin is the neural subtype of an adherens junction protein found in the apical region of premigratory NCCs, and plays an important role in controlling early phases of NCC migration. Although down-regulation of *αN-catenin* is critical for initial stages of NCC migration, the functional role of αN-catenin in later NCC migration and differentiation remains elusive. In this study, we investigate the spatio-temporal expression pattern of αN-catenin and elucidate effects on NCC movement and contribution to the trigeminal ganglia after perturbation of αN-catenin in the premigratory NCC population.

The role of the Adherens Junction protein αN-catenin in neural crest-derived Trigeminal ganglia formation

By

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Thesis submitted to the Faculty of the Graduate School of the

University of Maryland, College Park, in partial fulfillment

of the requirements for the degree of

Master of Science

2012

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Dedication

This thesis is dedicated in loving memory to my father, David William Hooper

and to my grandmother, Janice Pearl Rehrig, who have both shaped my life in

immeasurable ways.

Acknowledgements

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Chapter 1: Literature Review

1.1 Introduction

Early embryological development of a vertebrate organism is a highly dynamic yet tightly regulated process that ultimately will result in the formation of a highly structured, functioning organism from a single cell. To achieve this, cells must be directed to move in the appropriate spatio-temporal pattern and differentiate appropriately to build the embryo. Understanding key players, networks and mechanisms involved in the process of patterning cells is the broad and fascinating realm of developmental biology. Examining the role of an adherens junction protein, αN-catenin, in early vertebrate development is the focus of my thesis project, and I expect it will give further insight into the intricate cell-cell interactions that drive assembly of complex structures in the developing embryo

The chicken is a convenient model species for studying early vertebrate embryogenesis because of its relatively large size and accessibility due to external development in the egg. Additionally, recently developed methods allow for *in vivo* transgenesis, making it possible to study the roles of particular genes during development through gain-of and loss-of-function approaches (Sauka-Spengler and Barembaum 2008). For these reasons, many of the molecular pathways and regulatory networks in early vertebrate development, including those of the neural crest which are referred to in this study, have been elucidated with the help of the chick system. This model is broadly

applicable across vertebrate species because many of these mechanisms are

conserved in early development.

1.2 The Neural Crest

One cell type essential to vertebrate development is the neural crest, a transient, multipotent population of cells that originates at the border between the neural ectoderm and non-neural ectoderm in the developing embryo. As the germ layers are established during gastrulation, inductive signals from Bmps, Wnts, Fgfs, and Notch/Delta help establish the neural plate border and upregulate transcription factors in the Msx, Pax and Zic families at the neural-epidermal border (Hall 2008). As the invaginating neural ectoderm (Fig. 1, yellow) closes to form the neural tube (future central nervous system (CNS), premigratory neural crest cells remain in the dorsal neural folds (Fig. 1, blue) and extend along the rostrocaudal body axis (Sauka-Spengler and Bronner 2010). The Msx, Pax and Zic-family transcription factors will upregulate genes of the *Snail, SoxE, FoxD3* and other gene families that are specific to neural crest cells, activating downstream effector genes associated with migration and differentiation potency of neural crest cells (Hall 2008, Bronner and Ledouarin 2012). Sox10 in particular is a major player in several processes associated with neural crest cells including maintenance of multipotency, specification of neural crest cells into particular lineage fates, and the differentiation of specified cells. This transcription factor is expressed in early emigrating and migratory neural crest cells along the entire neural axis, but is rapidly downregulated in early stages of differentiation in many crest cells, making it a useful marker (Hall 2008).

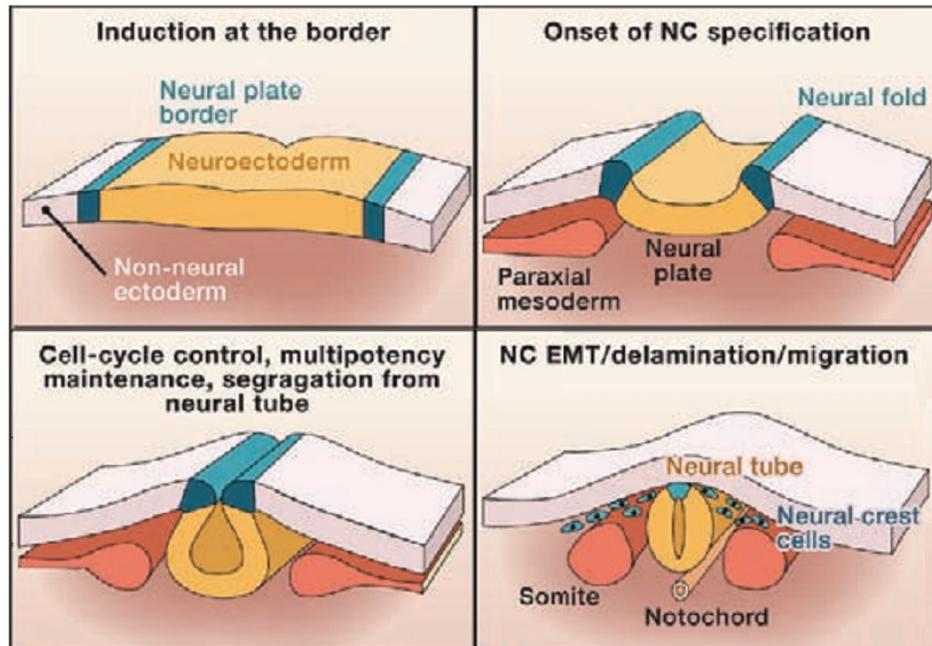**Figure 1 Neural Crest Cells** adapted from (Sauka-Spengler and Bronner 2010)

Neural crest cells (blue) are specified at the border of the invaginating neuroectoderm (yellow) and will remain in the dorsal region of the neural tube as it pinches off from the ectoderm (white). Neural crest cells will delaminate from the neural tube, gain migratory capacity, and migrate to various positions within the developing embryo.

Premigratory neural crest cells undergo a series of changes associated with the epithelial-to-mesenchymal transition (EMT) that underlies their transformation into a motile cell type. These include loss of cell-cell contact conveyed by adhesive junctions, breakdown of the basal lamina of the neuroepithelium, and loss of apicobasal polarity typical of epithelial cells (Hall 2008, Le Douarin and Kalcheim 1999). Snail2, Foxd3, Sox9 and Sox10 cause changes in cadherin-mediated adhesion with the downregulation of N-Cadherin first in the neural folds, followed by a transition to Cadherin6B expression, and finally a transition to Cadherin7 and 11 (Nakagawa and Takeichi 1995, Taneyhill 2008, Chalpe et al. 2010). The distinct spatio-temporal expression patterns of various cadherins in premigratory and migratory neural crest cells is thought to act as a cell sorting mechanism to prevent mixing of the populations of neural crest and non-neural crest cells while allowing crest cells to stay in contact via weaker adhesion of type II cadherins (Cadherins 7 and 11)(Theveneau and Mayor 2012).

Because neural crest cells are multipotent with stem cell-like properties, they have the ability to give rise to a variety of cell types and structures in the developing embryo. Once they have migrated to their final destination, neural crest cells can differentiate into muscle, portions of the heart, components of the gut and sensory nervous systems, pigment cells, and structures of the face, dependent upon molecular cues received from surrounding tissue and their axial level of origin (Hall 2008, Sauka-Spengler and Bronner 2010). For example, neural crest cells originating from the cranial region of the neural

tube may differentiate into bones and cartilage of the face, teeth, smooth

muscle, and cranial ganglia while those from the trunk will give rise to the

dorsal root ganglia of the spine, and melanocytes among other things (Fig. 2)

(Sauka-Spengler and Bronner 2010).

Improper regulation of neural crest cells at various time points in

development can lead to a variety of developmental defects collectively

termed neurocristopathies. These include craniofacial abnormalities,

Hirchsprung's disease (aganglionic megacolon) due to improper neural crest

cell migration to the gut, and neural crest cell-derived cancers, such as

melanoma and neuroblastoma (Fuchs and Sommer 2007). Additionally, neural

crest cells and some malignant cancers share similarities making the study of

neural crest cell EMT a good *in vivo* model for examining mechanisms of

metastasis. Many of the transcription factor families that are essential for

proper neural crest cell development are also upregulated in a number of

cancers, indicating some conserved regulation in these two models

(Theveneau and Mayor 2012). Therefore, understanding the molecular

aspects of neural crest cell migration and differentiation under normal

conditions becomes a vital tool for informing our knowledge of the diseased

state and for developing potential therapeutics for the treatment of human
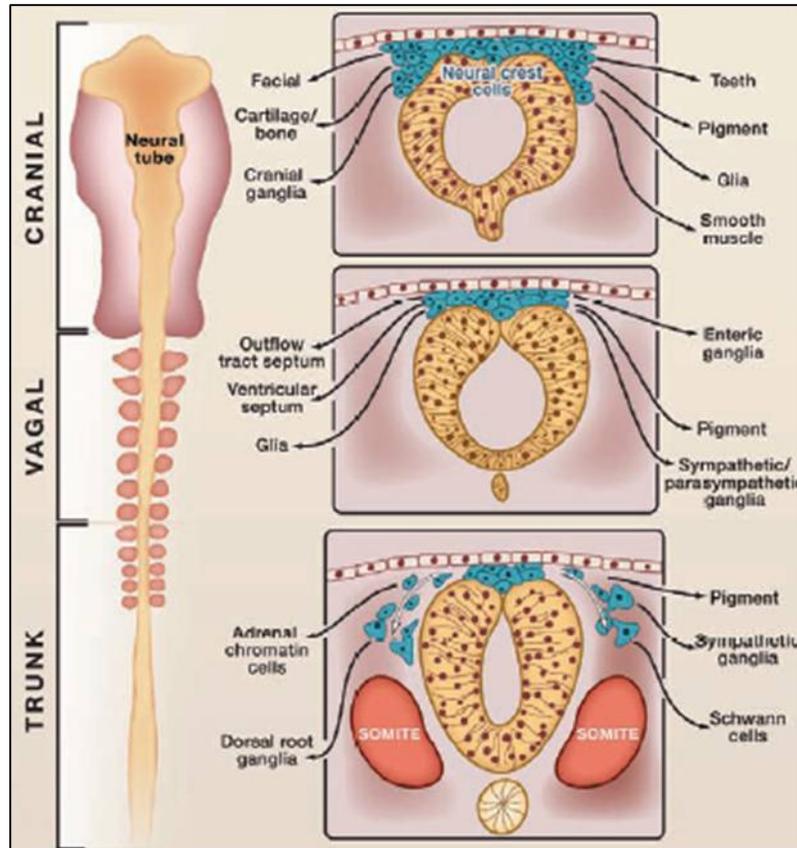
diseases and disorders.

Figure 2 Neural crest cell derivatives adapted from (Sauka-Spengler and Bronner 2010)

This illustration shows the many and varied derivatives of neural crest cells at multiple axial levels. Cranial ganglia, as well as the various structures of the vertebrate face, derive from the cranial neural crest cells.

1.3 Cranial Ganglia: Neural Crest and Placode Interactions

In vertebrates, sensory neurons of the peripheral nervous system are responsible for relaying somatosensory (touch, pain, temperature) information from the external environment to the CNS. The dorsal root ganglia perform this function in the trunk and originate solely from trunk neural crest cells. Cranial ganglia on the other hand, including the trigeminal (V), facial (VII), glossopharyngeal (IX), and vagal (X) cranial nerves, are comprised of a dual population of cells. Using embryonic tissue transplantation from quail and chicken (which are histologically distinct), D'Amico-Martel and Noden confirmed the dual origin of various cranial ganglia from cranial neural crest cells and neurogenic ectodermal placodes, including both lobes of the trigeminal (V) cranial nerve, (D'Amico-Martel and Noden 1983).

Similar to neural crest cells, epidermal sensory placodes arise at the border of the developing neural plate in distinct rostro-caudal positions. These paired localized ectodermal thickenings in the vertebrate head give rise to cranial sense organs, such as the ear and nose, and contribute to the cranial sensory nerves (Baker and Bronner-Fraser 2001) and (Graham and Begbie 2000) and (Park 2010)(Fig.3). By using Pax3 as a marker for the neurogenic ophthalmic placode and implanted barriers between the neural tube and ectoderm, it was shown that a diffusible signal from the neural tube (neuroectoderm) is required for placodal induction and maintenance (Stark et al, 1997). Furthermore, this group showed that neural crest cells are not

necessary to induce *Pax3* expression nor invagination of the neurogenic

placodes, but that absence of
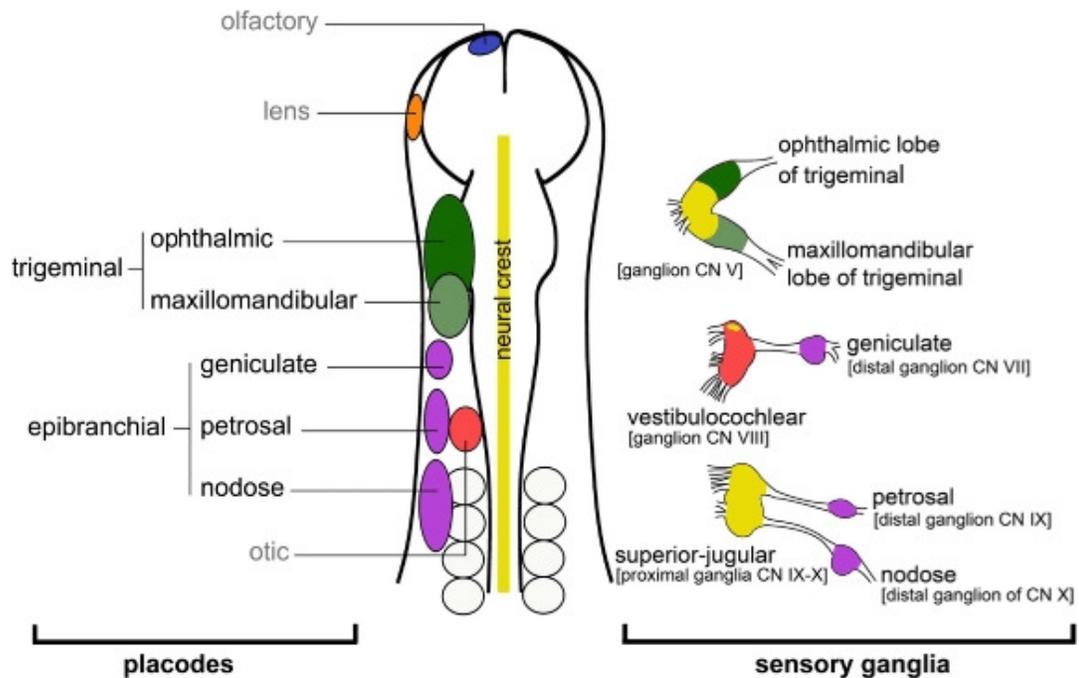
Figure 3 Placodal fate map and derivatives adapted from (Park 2010).
Schematic illustrating the fate map (left side) and derivatives (right side) of cranial placodes in the chick embryo. Distinct placodal regions in the embryonic ectoderm contribute sensory neurons to the distal lobes of the trigeminal ganglion (V) and the distal ganglia of cranial nerves VII, IX, and X. The neural crest (yellow) contributes sensory neurons to the proximal trigeminal ganglion (V) and the proximal

neural crest cells due to ablation leads to improper localization and poor

condensation of *Pax3-* expressing ganglia (Stark et al. 1997). The exact

molecular components of the diffusible signal from the neural tube have not

yet been elucidated; however, reverse transcriptase-PCR screening for

receptors expressed by uninduced placodal ectoderm has shown the

presence of receptors for fibroblast growth factors, insulin-like growth factors,

platelet-derived growth factors, Sonic hedgehog (Shh) and Wnts (McCabe,

Shiau and Bronner-Fraser 2007), thereby indicating potential interactions

involved in placodal induction.

The trigeminal ganglia are a bi-lobed subset of the cranial sensory

ganglia that transmit somatosensory stimuli from the face, jaws and teeth. The

trigeminal ganglia consist of the molecularly distinct maxillomandibular and

ophthalmic lobes and provide a convenient model for studying the cell-cell

interactions necessary for the normal formation of cranial ganglia due to their

large size and accessibility (Begbie, Ballivet and Graham 2002). The proximal

regions of these ganglia, as well as all glial cells that envelop their axons, are

of neural crest cell origin, while the placodes contribute to more distal portions

(D'Amico-Martel and Noden 1983). The proper intermixing of these dual

populations relies, not surprisingly, on complex molecular interactions. As well

as being a presumptive inducer of the trigeminal placodes, Shh has been

implicated as a regulator of the movement of trigeminal placode cells, as

migration of trigeminal placodal cells is halted by local recombinant Shh, and

absence of Shh in mouse embryos causes lateral placode cells to migrate to

the ventral midline (Fedtsova, Perris and Turner 2003). Neuropilin-2/Semaphorin-3F signaling was additionally found to be essential for cranial neural crest cell migration and formation of the trigeminal ganglia in the mouse and chick models (Gammill, Gonzalez and Bronner-Fraser 2007). *Neuropilin-2* and *Semaphorin-3F* knockout mice have defects in ganglion coalescence ranging from mild to severe, indicating that both signals of the receptor/ligand pair are required to pattern neural crest cell migration via repulsive cues (Gammill et al. 2007). Further examination of molecular cues showed that ingressing placode cells from the ectoderm express the Robo2 receptor while the migrating cranial neural crest cells express the Robo2 ligand Slit1, indicating a potential mechanism for these two populations to intermix appropriately. Depleting either of these proteins leads to abnormal neural crest-placode interactions and results in abnormal ganglion assembly, indicating a possible role for neural crest cells as a scaffolding in ganglion assembly to allow for appropriate condensation of cells (Shiau et al. 2008).

Proteins that modulate cell-cell interactions frequently play roles in many structures in higher vertebrates, and this is also the case of the cranial ganglia. In the zebrafish model, Cadherin-2 (also known as N-Cadherin) is expressed in cranial ganglia and the lateral line system and plays important roles in development, as morpholino-mediated knockdown in the early embryo leads to severely disrupted ganglion formation (Kerstetter et al. 2004). N-Cadherin in zebrafish cranial sensory ganglia has been shown to act cell autonomously and is essential for axon pathfinding in the sensory ganglia

(LaMora and Voigt 2009). More recently in the chick model, N-cadherin has been shown to be expressed on the surface of placodal cells as an important mediator of coalescence, as depletion of N-cadherin in the placodes causes poor trigeminal ganglia assembly (Shiau and Bronner-Fraser 2009). Likewise, adjusting expression levels of Slit-Robo in the NEURAL CREST CELLs or placode cells modulates N-Cadherin expression post-translationally in the chick, indicating a mechanism by which N-Cadherin acts in concert with Slit-Robo signalling (Shiau and Bronner-Fraser 2009).

1.4 Cadherins/Catenins in development and in Neural crest cells

As mentioned previously, adherens junctions and their components play crucial and varied roles in tissue structure and morphogenesis throughout early embryonic development. These include modulating cell-cell adhesions, coordinating movement via the actin cytoskeleton, and involvement in multiple signaling pathways (Stepniak, Radice and Vasioukhin 2009) and (Meng and Takeichi 2010). Adherens junctions are formed by complex and dynamic interactions between the Cadherin and Catenin families of proteins and subsequently link the adhesive function of these complexes to the actin cytoskeleton (Stepniak et al. 2009). Cadherins are calcium-dependent transmembrane proteins that have an extracellular domain comprised of five cadherin motifs and a cytoplasmic domain with two conserved motifs (Hulpiau and van Roy 2009). Some members of the Cadherin family include E-(epithelial) and N- (Neural) cadherin, among others, and these cadherins typically implement cell-cell adhesion by binding to each other in a homotypic fashion. Intracellularly, cadherin proteins interact both directly and indirectly with catenin molecules (Stepniak et al. 2009)(Fig. 4). Catenins, including the Wnt-signaling molecule β-catenin and the actin-binding protein α-catenin, are crucial to the normal function of the adherens junction in cellular adhesion. Alpha(α)-catenin was initially identified in 1991 as a 102 kD protein associated with calcium-dependent adhesion molecules and acknowledged to be a component of the adherens junction. Additionally, it was shown that in cadherin-negative cells, α-catenin was found distributed in the cytoplasm,
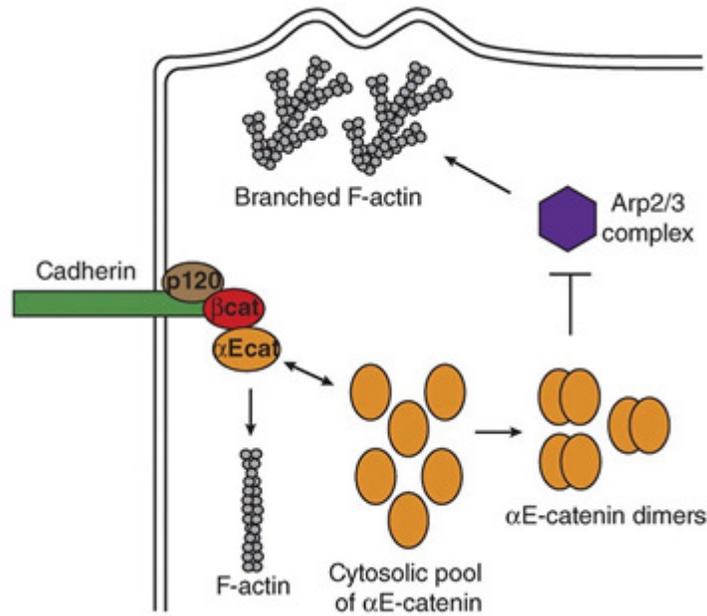
Figure 4. Cartoon diagram of the adherens junction adapted from (Maiden and Hardin 2011)
This model illustrates two distinct populations of α-catenin. At the adherens junction, α-catenin associates with β-catenin of the Cadherin-catenin complex (CCC) and mediates attachment to the actin cytoskeleton. Close to the membrane, a cytosolic pool of α-catenin is created through dissociation from the CCC and is able to homodimerize. These α-catenin homodimers inhibit the Arp2/3 complex and thereby regulate membrane dynamics by preventing the formation of branched F-actin.

suggesting potential alternative functions of this protein (Herrenknecht et al. 1991; Nagafuchi, Takeichi and Tsukita 1991). Analysis of a human lung cancer line (PC9) with dysfunctions in cadherin-mediated cell-cell adhesion despite the presence of E-cadherin showed the adhesive dysfunction was due to impaired production of α-catenins in the cells (Shimoyama et al. 1992). These results highlight the importance of this junctional molecule in forming a functional adherens junction.

The previously accepted model of the adherens junction was based upon a quaternary structure in which α-catenin can bind both both β-catenin and actin through separate binding domains, thus linking cell-cell adhesion to the actin cytoskeleton(Gates and Peifer 2005). Recent biochemical and structural data, however, does not support this model, as it was found that the binding of α-catenin to β-catenin prevents α-catenin from binding actin at the same time (Pokutta et al. 2008, Scott and Yap 2006). In this newer model, the pool of α-catenin recruited to the membrane will form homodimers upon dissociation from the cadherin-β-catenin complexes and stabilize the actin cytoskeleton by suppressing Arp2/3-mediated actin branching (Pokutta et al. 2008). As well as its well-established role in adhesion, α-catenins have been explored as modulators of signaling, specifically in antagonizing Wnt signaling, due to their ability to bind to β-catenin (Sehgal, Gumbiner and Reichardt 1997).

αN-catenin was identified in 1992 as a protein associated with chicken N-cadherin and is the neuronal subtype of the alpha-catenins, with 81.6%

similarity to the originally discovered α-catenin (Hirano et al. 1992). Again examining the PC9 lung carcinoma cells with impaired adhesion, transfection with αN-catenin allowed these cells to gain adhesive abilities and induce a polarized phenotype, showing that αN-catenin is a critical mediator of cadherin function and in implementing multicellular structure [(Hirano et al. 1992) and (Watabe et al. 1994)]. Not long after identifying this protein and realizing its developmental significance, the expression patterns of αN-catenin and N-cadherin in the chicken were examined. αN-catenin was expressed in a tissue-specific manner, most highly in the nervous system, but not solely in association with N-cadherin (Hirano and Takeichi 1994). Further examination of this novel protein in the mouse resulted in the identification of two isoforms of αN-catenin, both being expressed strongly in the nervous system, especially within axonal fibers of neurons (Uchida et al. 1994). Similarly in the rat model, Stocker and Chenn examined the expression pattern of αN-catenin in the CNS, showing in culture that it was expressed in both neural precursors as well as differentiated neurons (Stocker and Chenn 2006). In the chicken, studies have shown that αN-catenin is expressed in the apical region of the neural tube, with diminished αN-catenin levels dorsally upon NEURAL CREST CELL emigration from the dorsal neural tube (Jhingory et al. 2010)(Fig. 5). In the adult vertebrate brain, αN-catenin colocalizes with the synaptic marker synaptophysin and is present in the synaptic junctions of neurons (Uchida et al. 1996).
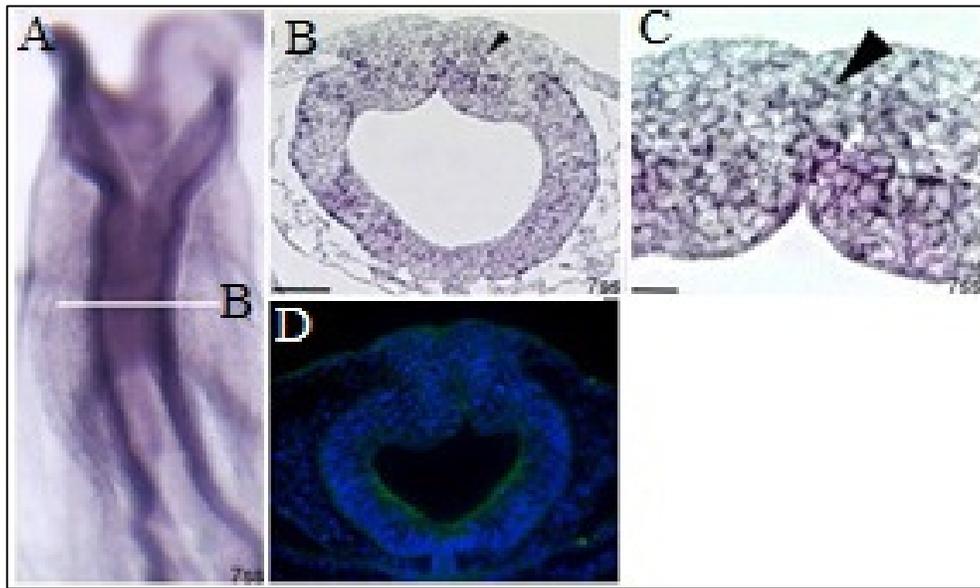
Figure 5. αN-catenin is localized to the apical neural tube and diminished dorsally upon neural crest emigration.
adapted from (Jhingory, Wu and Taneyhill 2010)
Whole-mount (A) and transverse sections (B-D) of αN-catenin transcript (B,C) and protein distribution (D, green) in the chick embryo midbrain. αN-catenin is down-regulated upon NCC delamination from the neural tube (see arrowhead marking delaminating NCCs (B,C). Likewise the protein product is decreased in the dorsal region of the neural tube (D).

Based on the presence and localization of this junctional protein throughout development and in various vertebrate model systems, it is not surprising that αN-catenin misexpression would have effects on embryonic development. In mice that carry a deletion in the *αN-catenin* gene, Purkinje cells of the brain will occasionally localize abnormally and will have loosened adhesion in cells that do reach their targets (Park et al. 2002). Further studies using two different lines of *αN-catenin*-deficient mice displayed abnormal axon pathfinding and arrangement of neuronal structures in the brain (Uemura and Takeichi 2006). In the chicken, knock-down and overexpression of αN-catenin via electroporation into the neural tube has immediate effects on neural crest cell delamination leading to enhanced and inhibited neural crest cell migration, respectively, due to effects on Cadherin6B (Jhingory et al. 2010).

In the following chapters, I will use the chick as a vertebrate model to examine αN-catenin expression in the region of the neural crest-derived trigeminal ganglia. In addition, I will determine effects of its misexpression on the neural crest cell contribution to the forming trigeminal ganglia as well as effects on proper ganglia morphology.

Chapter 2: Materials and Methods

Chicken Embryos

Fertilized chicken eggs (*Gallus gallus)* were obtained from B&E and Hy-Line North America, L.L.C., and incubated at 37°C in humidified incubators. Embryos were staged according to either the number of somite pairs (somite stage, ss) in early development or Hamburger and Hamilton (HH) in later development (Hamburger and Hamilton 1992).

Electroporation and knock-down of *αN-catenin* using an antisense morpholino *in vivo*

A 3' lissamine-labeled antisense *αN-catenin* morpholino (MO) was used for knock-down experiments. This MO was designed according to manufacturer's criteria and targets *αN-catenin* mRNA, effectively disrupting translation. The sequence for the *αN-catenin* MO is 5'-CGTTGCAGAAGTCATACTCCCTCA-3' and for the 5 base pair mismatch *αN-catenin* control MO is 5'-CcTTcCAGAAcTCATAgTCCgTCA-3' (mutated bases are in lower case; GeneTools, L.L.C.). MOs were introduced into the early chicken neural tube by fine glass needles at the 2-5ss prior to neural crest cell EMT and migration to target the neural crest cells on one side of the dorsal neural tube. After filling the neural tube, platinum electrodes were placed on either side of the embryo, and two 25 volt, 25 millisecond electric pulses were applied across the embryo in order to electroporate one side of the neural tube only as in (Itasaki et al., 1999) and (Jhingory et al., 2010). Eggs were re-sealed with tape and parafilm

and re-incubated for 14-16 hours, then imaged *in ovo* prior to embryo turning around HH13 using a Zeiss AxioObserver.Z1 microscope in order to determine electroporation efficiency. After imaging, eggs were re-sealed and re-incubated for the desired time period.

Overexpression of *αN-catenin* in vivo

Full-length *αN-catenin* cDNA previously cloned into the pCIG chick expression construct in the Taneyhill lab was used to overexpress *αN-catenin in vivo* (Jhingory et al. 2010). The pCIG-*αN-catenin* or control (pCIG) vector at a concentration of 2.5 µg/µl was electroporated in the early chicken neural tube as described above.

Whole-mount *in situ* hybridization

Whole-mount *in situ* hybridization was performed to detect mRNA transcripts of *Sox10* and *αN-catenin* as previously described in (Jhingory et al. 2010) and (Wilkinson and Nieto 1993). Briefly, digoxigenin-labeled riboprobes were created from linearized cDNA of the gene of interest, which had previously been cloned. For older embryos, treatment with Proteinase K was performed to increase accessibility of the probe to target RNA. Stained embryos were imaged in 70% glycerol using a Zeiss SteREO Discovery V8 microscope with a mounted camera. Transverse sections were obtained by embedding embryos in gelatin and cryostat-sectioning at 14 µm on a Leica Jung frigocut 2800E. Sectioned images were taken using a Zeiss AxioObserver.Z1 microscope and processed using Adobe Photoshop 9.0 (Adobe Systems).

Immunohistochemistry and TUNEL assay

Immunohistochemical detection of TuJ1 (Covance, 1:200) was performed in whole-mount following 2 hour fixation in 4% PFA. Immunohistochemical detection of HNK1 (1:100), N-cadherin (Developmental Studies Hybridoma Bank (DSHB) clone Ncd21, 1:200), Islet-1 (DSHB, clone 40.2D6, 1:250), phospho-histone H3 (Millipore,1:200) and GFP (Invitrogen,1:250) was performed on 14 µm transverse sections following 4% PFA fixation and gelatin embedding, and αN-catenin (DSHB, clone NCAT2; 1:100) was carried out on transverse sections following methanol fixation and gelatin embedding. All primary and secondary antibodies were diluted in 5% sheep serum in 0.1% Triton-X or 0.1% Tween-20 in PBS. The following secondary antibodies were obtained and used at 1:200 or 1:500 dilutions in 5% sheep serum: Alexafluor 488/594 goat anti-mouse IgM, Alexafluor 647 goat anti-mouse IgG1, Alexafluor 488 donkey anti-rabbit IgG, and Alexafluor 594/647 goat anti-rat. TUNEL assay (Roche, TMR red and fluorescein) was performed on 4% PFA-cryopreserved sections to detect apoptotic cells. Sections were stained with 4',6-diamidino-2-phenylindole (DAPI) to mark the cell nuclei, and sectioned images were captured and imaged as described above.

Cell Counting, Quantification and Analysis

Islet-1 positive cells were counted individually following immunohistochemistry using the Adobe Photoshop count tool and analyzed in Microsoft Excel to compare cell numbers on the treated side versus the contralateral control side.

HNK1 area of the treated side was quantified using Photoshop software to

calculate arbitrary square pixel units of sections at the same magnification

(20x) to compare against the HNK1 area of the contralateral control side.

Results were analyzed with a Student's t-test at a significance level of .05.

Results

Chapter 3: αN-catenin transcripts and protein are present in later stage chicken embryos in the trigeminal ganglia

Neural crest cells and placodal cells that contribute to the forming trigeminal ganglia must undergo several distinct steps throughout gangliogenesis to gain the ability to migrate into the mesenchyme and comingle to form the final product. Initially, both cell populations must undergo an epithelial-to-mesenchymal transition to delaminate from the ectoderm and dorsal neural tube epithelial layers. Next the cells migrate individually to the region of ganglion formation where the two populations will interact and condense to form the highly structured trigeminal ganglia. Several attractive and repulsive guidance cues have been implicated in the roles of neural crest migration along all axial levels including the repulsive cues of Eph-ephrins in trunk neural crest cells (Kasemeier-Kulesa et al. 2006), neuropilin-Semaphorin in cranial neural crest cells (Gammill et al. 2007) and laminin- extracellular matrix interactions in both cranial and trunk neural crest cells (Coles, Taneyhill and Bronner-Fraser 2007). More specifically, recently studied mechanisms of cranial ganglia formation include Robo2-Slit1-mediated attraction between placodal and neural crest populations (Shiau et al. 2008) and N-Cadherin-mediated coalescence of the dual cell populations (Shiau and Bronner-Fraser 2009). These studies implicate the necessity of cell surface receptors and

proper regulation of adhesion molecules in the normal process of neural crest cell migration and formation of their final derivatives.

αN-catenin has been shown to be down-regulated in premigratory and newly emigratory neural crest cells by the transcriptional repressor Snail2 (Jhingory et al. 2010). The first aim of this study is to examine the presence or absence of αN-catenin in later stages (HH16-17) in the cranial neural crest cells that will contribute to the trigeminal ganglia. This was initially done by detecting mRNA transcripts by *in situ* hybridization in the region of the condensing ganglia. Presence of transcripts was difficult to determine in whole-mount embryos likely due to poor penetration and low abundance (whole mounts not shown) but was detected on 14μm sections of an HH16 embryo (Fig. 6, A and D). Subsequent immunohistochemistry for HNK1, a cell surface antigen commonly used as a marker for neural crest cells (Hall, 2009), was performed on sections (Fig. 6, B and E). Results show the presence of *αN-catenin* transcripts in later migratory neural crest cells, prior to trigeminal ganglia formation, as seen by co-localization in the merge image (Fig. 6, C and F). The region of the condensing ganglia is outlined in Fig. 6E.
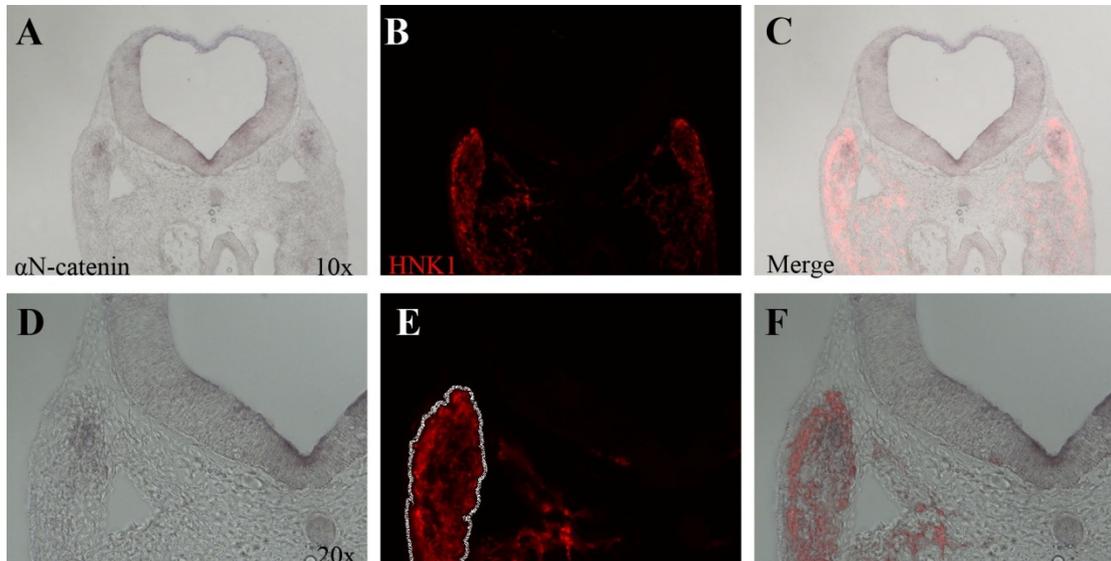
Figure 6. Transverse sections through the trigeminal ganglia region in the chick embryonic head show colocalization of αN-catenin transcripts and HNK1-expressing neural crest cells. (A, 10x magnification)αN-catenin *in situ* hybridization was performed to detect the presence of transcripts in older embryos. (D, 20x) Magnified field of the neural tube and ganglia region. (B, E) Subsequent immunohistochemistry for HNK1 was performed on these sections to detect migratory and condensing neural crest cells. (C, F) Merge of images show the colocalization of αN-catenin transcripts with HNK1 staining, indicating αN-catenin is being transcribed in migratory neural crest cells, notably in the region of the condensing trigeminal ganglia as outlined in (E).

Next we examined the presence of αN-catenin protein by immunohistochemistry on sections in HH17 embryos as the dual populations begin to condense into the structures of their final derivatives (Fig. 7, 10x and 40x). At this magnification, αN-catenin appears to be re-expressed in the migrating neural crest cells as expression co-localizes with HNK1 (Fig. 7, D and H, merge image).

These results indicate that αN-catenin is in fact re-expressed in later migratory neural crest cells in the proper time and place to be a potential mediator of ganglion coalescence within the neural crest cell population. Its presence in the cytoplasm of some mesenchymal cells and may be explained by possible roles of αN-catenin apart from adhesion, such as modulating cell migration and cell signaling. In the following chapters, studies will examine the effects of long-term perturbation of αN-catenin specifically in the neural crest cell population.
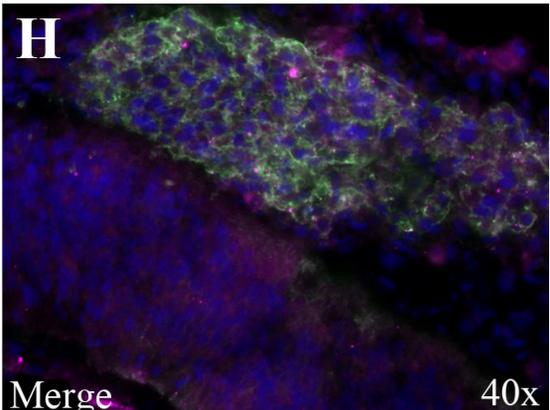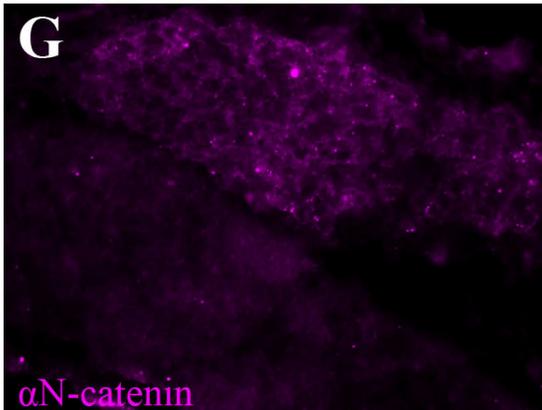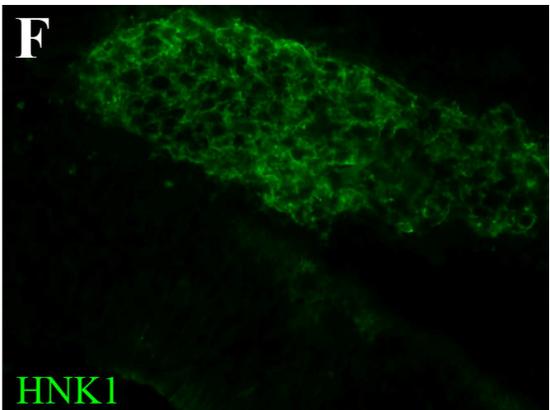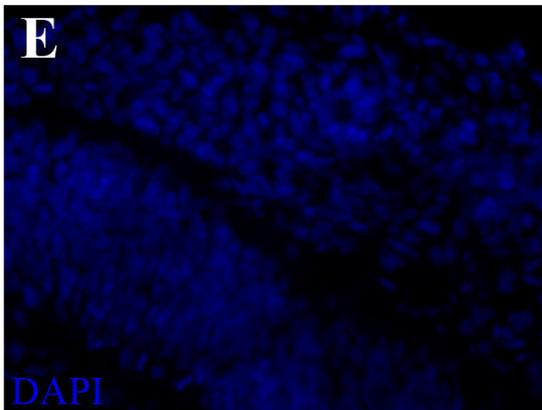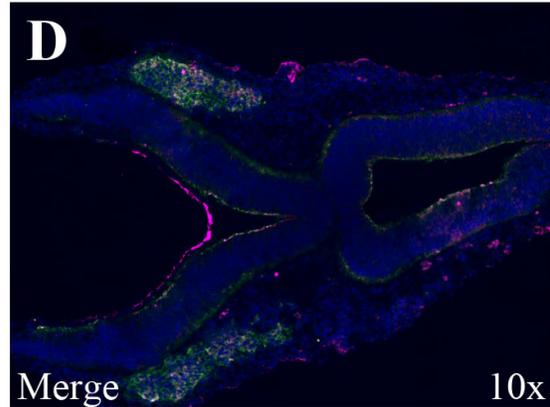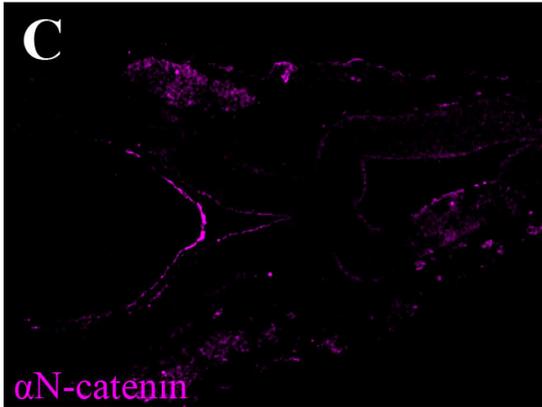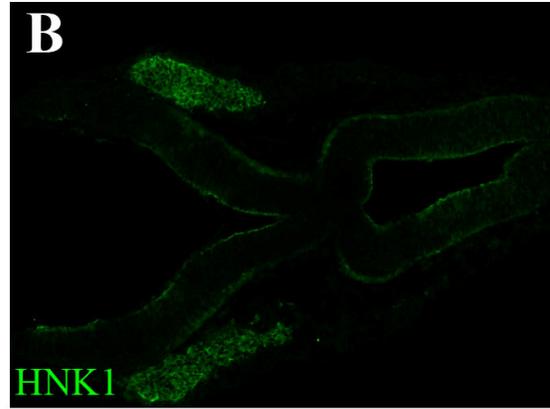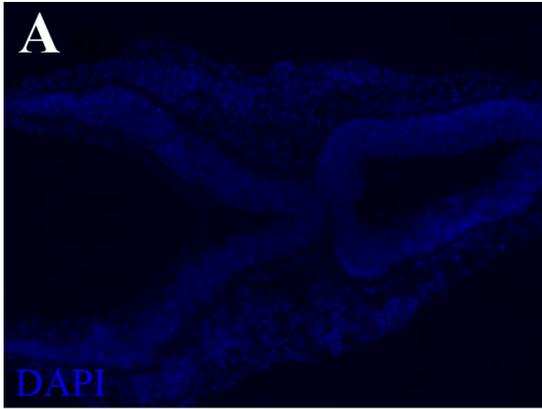
Figure 7. αN-catenin and HNK1 dual immunohistochemistry show protein colocalization. (A-D) Transverse section through the trigeminal ganglia region of an HH17 chick embryo at 10x. (E-H) Higher magnification of the trigeminal ganglia. (A and E, blue) DAPI staining was performed to mark cell nuclei. (B and F, green) HNK1 protein was used as a neural crest cell marker. (C and G, magenta) αN-catenin protein was detected in the region of the condensing trigeminal ganglia. (D and H)Merged image shows the colocalization of αN-catenin protein in the neural crest cells.

Chapter 4: Effects of αN-catenin knockdown in the neural crest cell population

***4.1 In ovo* imaging allows us to detect successful electroporation of a lissamine-tagged morpholino.**

To knock-down αN-catenin expression, a morpholino antisense oligonucleotide was injected and into the chick neural tube at the 2-5 somite stage (ss), prior to neural crest cell migration, and electric pulses were used to transfect one side of the neural tube only. To circumvent difficulties imaging whole embryos after they undergo stereotypic rightward torsion within the egg at around HH13, a method of *in ovo* imaging was adopted to detect electroporation efficiency at 12-18 hours post-electroporation (Fig. 8C, see Materials and Methods for more details). In this way, we were able to see at earlier stages that the correct side of the embryo was electroporated with the morpholino (Fig. 8C, arrow). The embryos were then allowed to re-incubate for specified time periods and were subsequently processed to determine effects on the final localization of neural crest cells as well as the structure and placodal contribution to the condensing trigeminal ganglia.
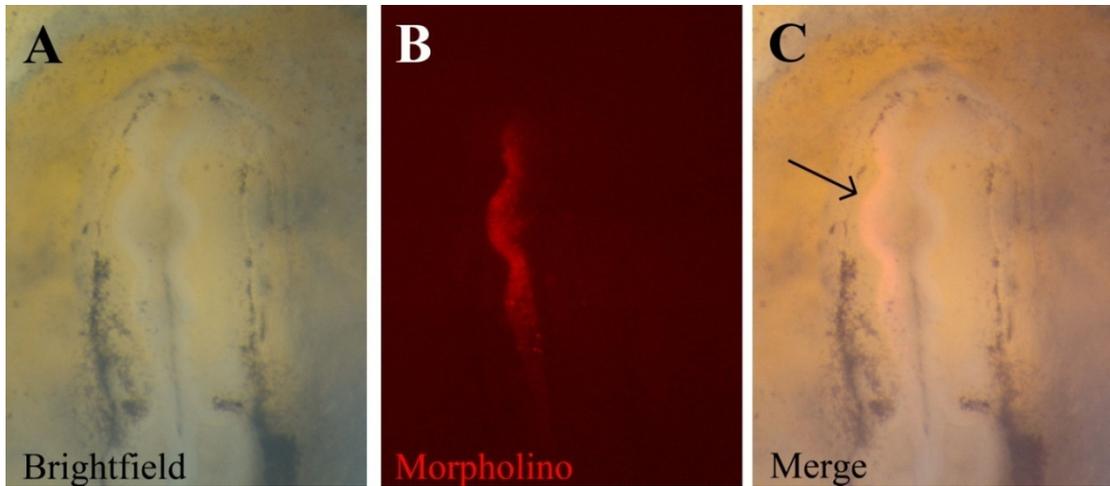
Figure 8. *In* **ovo imaging allows us to determine embryos that have been successfully electroporated while allowing for further development.** (A) Brightfield image at 40x magnification to show the outline of the neural tube of the developing embryo 15 hours post-electroporation. (B) Detection of the lissamine (red)-tagged morpholino shows the presence of morpholino in the embryo at this stage. (C) Merge image allows us to determine that embryos were successfully electroporated on the left side of the neural tube (arrow) and re-incubate an additional amount of time.

4.2 αN-catenin knockdown expands the neural crest domain in early stages consistent with previous findings, but does not result in significant changes to neural crest cell contribution to the trigeminal ganglia at the time of condensation

Whole-mount *in situ* hybridization was performed after electroporation on the left side of the developing embryo, and embryos were collected at several time points in order to assess effects on the migratory neural crest cell population after αN-catenin depletion. Upon detection of *Sox10* transcripts, which mark migratory neural crest cells, in whole-mount, the embryos were sectioned to measure the migratory neural crest cell domain to compare against the contralateral control side (Fig. 9). Whole-mount images (Fig. 9, A,B arrows) reveal a denser area of neural crest cells in the head region upon αN-catenin depletion when compared to the contralateral control side of the embryo, with no change observed upon treatment with the Control MO (Fig. 9F-J). Moreover, serial sections through the head region in (Fig 9C-E) show an increase in the dorsal-ventral domain occupied by migratory neural crest cells on the αN-catenin-depleted side (Fig 9, asterisk in C), with no change observed in the control (Fig.9H). These findings are consistent with previously published results that early αN-catenin depletion in the neural crest cells leads to premature and enhanced neural crest cell migration (Jhingory et al. 2010).
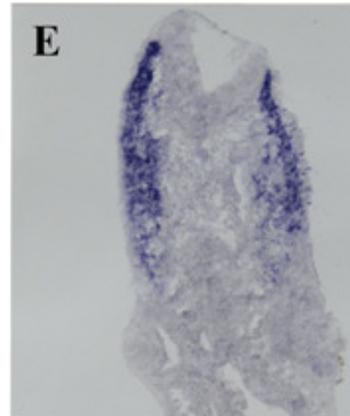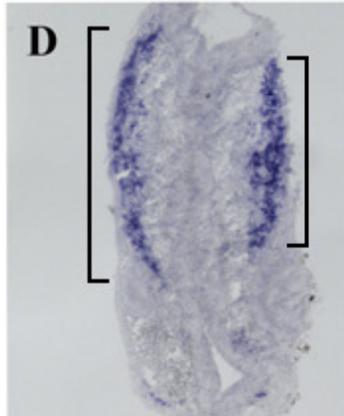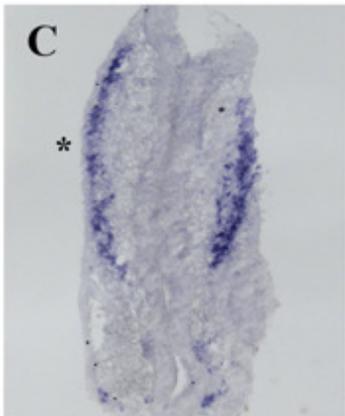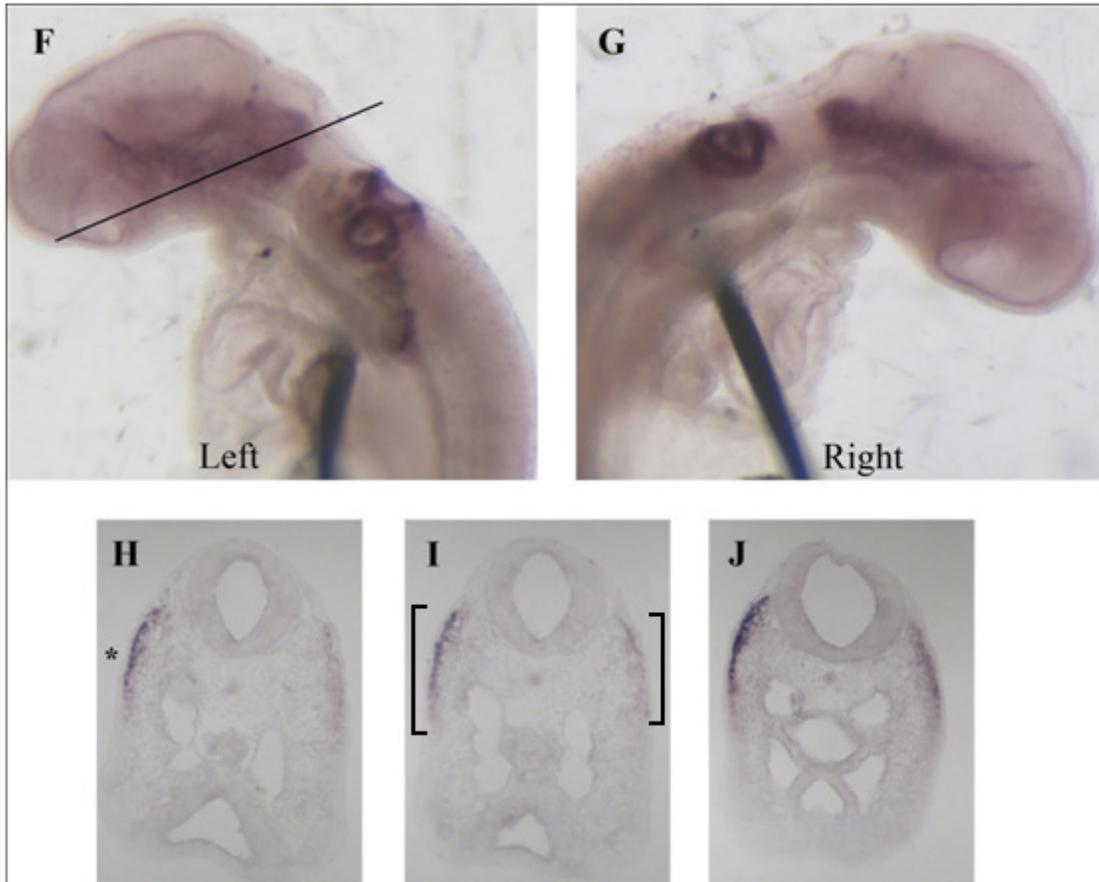
Figure 9. Detection of *Sox10* transcripts after αN-catenin knockdown on the left side shows increased size of the neural crest cell domain as compared to control embryos. (A,B) Brightfield images of a whole mount embryo electroporated with αN-catenin MO on the left side and processed to detect *Sox10* transcripts after 27 hours of incubation. (C-E) Serial sections through the head of the embryo through the migratory neural crest cell population in the plane indicated by the black bar in (A) show an expanded domain on the left side (asterisk in C). (F,G) Brightfield images of a whole-mount embryo electroporated with Control MO on the left side and processed to detect *Sox10* transcripts. (H-J) Serial sections through the head of the embryo through the migratory neural crest cell population in the plane indicated by the black bar in (F) show consistent domain sizes, though lighter staining on the right could be due to poor riboprobe penetration or uneven Proteinase K treatment.

HNK1 was used as another marker to detect migratory neural crest cells via protein localization using immunohistochemistry (Fig. 10). Two embryos sectioned through the trigeminal ganglia of the head at HH17 (Fig. 10A-D and E-F) show the trigeminal ganglia that is condensing and forming lobular structures (outlined in Fig. 10F). The condensing ganglia on the electroporated side appear objectively larger (Fig. 10B,F). Measurements to quantify the area of the condensing trigeminal lobes were performed in Photoshop and analyzed with a Student's t-test and found to be not significantly different than the area of the contralateral control (Fig. 10I). In this way, the early effects of αN-catenin depletion on neural crest cell migration appear to be abrogated by the time they reach the stage of condensation, with no statistical differences in neural crest cell contribution detected.
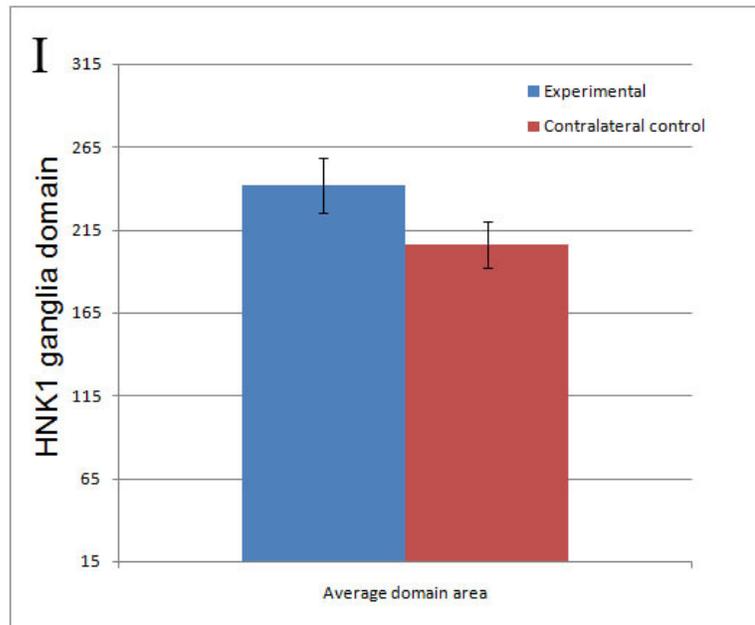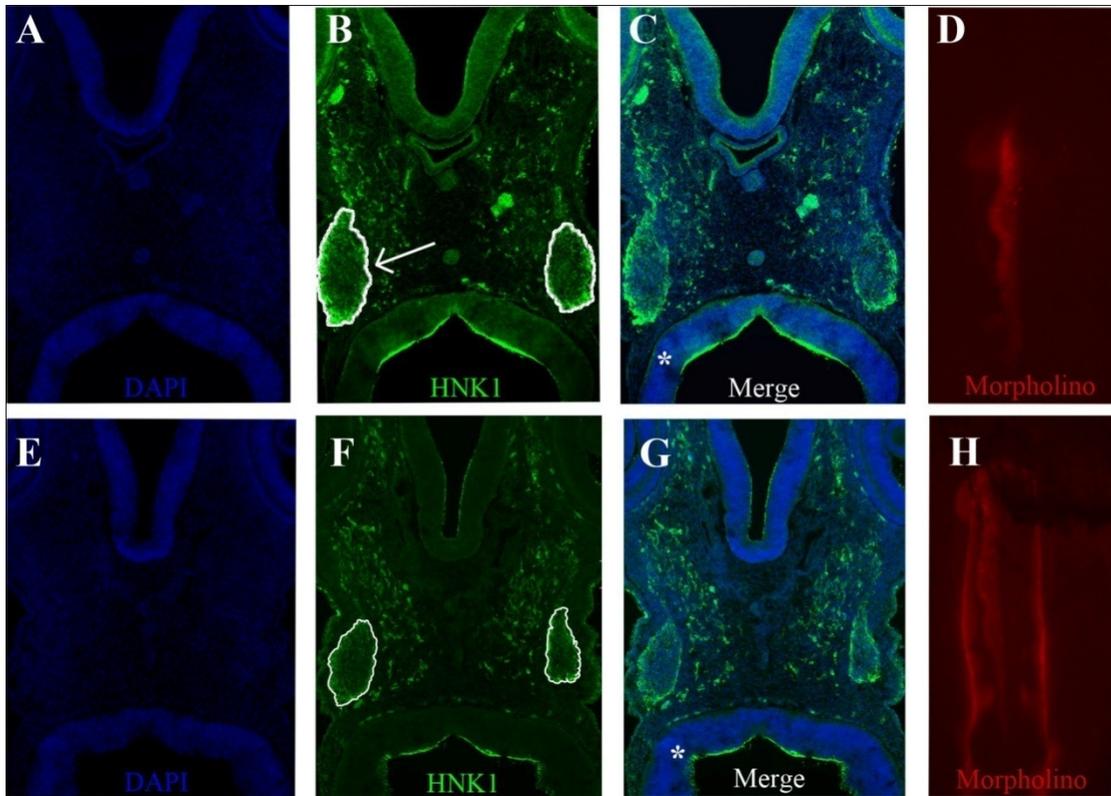
Figure 10. Detection of HNK1 protein by immunohistochemistry after αN-catenin knockdown shows the neural crest domain size of the condensing trigeminal ganglia. (A-D) and (E-H) are sections through the head of two separate embryos electroporated on the left side of the neural tube and re-incubated a total of 38 hours to reach HH17. Fluorescent images were taken at 10x magnification of DAPI to mark cell nuclei (A,E, blue) and HNK1 (B,F, green, arrow in B). (C and H) Merge image shows the location of neural crest cells in the embryo. The trigeminal ganglia are condensing at this stage and are outlined in (F). (I) Quantification of ganglion areas are shown as arbitrary square pixel units, and are not statistically different.

4.3 αN-catenin knockdown in the neural crest cell population leads to disrupted trigeminal ganglia structures but no significant changes in placode contribution to the trigeminal ganglia

Because the trigeminal placode arises from a dual contribution of neural crest cells and placode cells, we sought to examine potential effects of αN-catenin knockdown in neural crest cells on developing placode cells. To reveal the structure of the developing ganglia in whole-mount embryos, an antibody to β-neurotubulin (TuJ1) was used to detect differentiated neurons. At the stages measured (HH16-17), the majority of the differentiated neurons will be from the placodal population (Shiau et al. 2008). Using this marker and imaging the embryonic head, we can then detect morphological differences from the electroporated side and compare against the contralateral control (Fig. 11). Knockdown of αN-catenin in the premigratory neural crest cell population resulted in disruption of the trigeminal ganglia with phenotypes ranging from mild (Fig. 11A) to severe (Fig. 11C,E).

To further explore the possibility that αN-catenin knockdown in the neural crest cell population has long-term effects on placode aggregation in the dual population-derived trigeminal ganglia, we used Islet-1 to mark placode cells that contribute to both lobes of the trigeminal ganglia (Fig. 12). Dual immunohistochemistry with HNK1 allows us to examine the complex intermixing of Islet-1-positive placodal cells with HNK1-positive neural crest cells in the condensing ganglia of the merge image (Fig.12D,H,K).

Experimental **Contralateral Control**

Mild

A — TuJ1
B — TuJ1

Severe

C — TuJ1
D — TuJ1

Severe

E — TuJ1
F — TuJ1
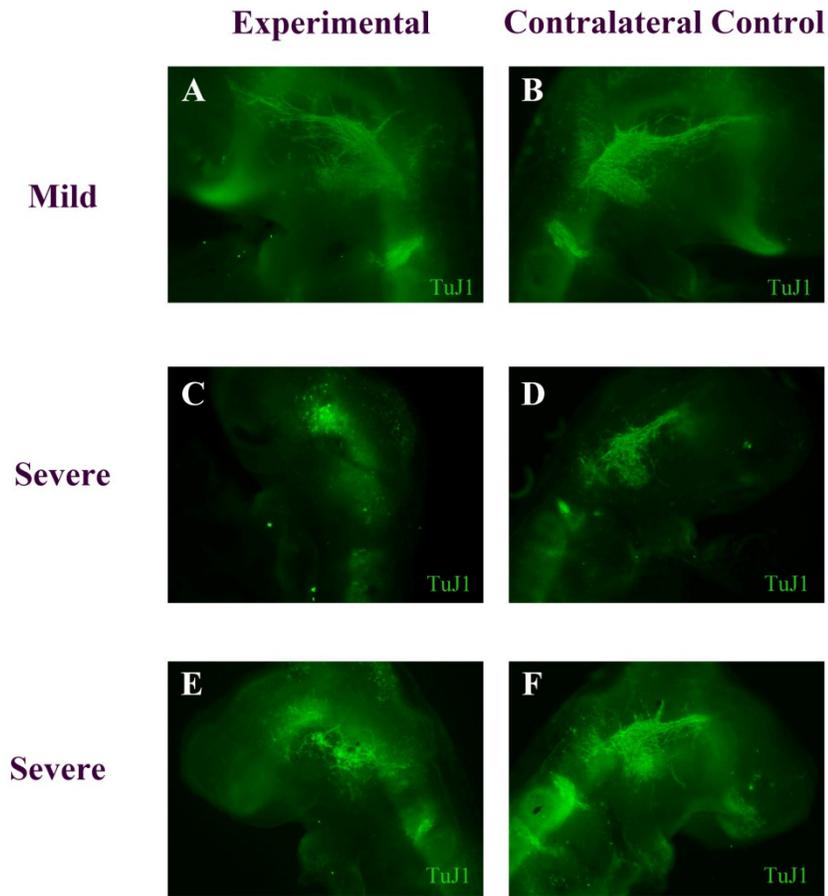
Figure 11. αN-catenin knockdown disrupts the structure of the forming trigeminal ganglia. (A-F) Representative examples of 3 embryos electroporated on the left side of the neural tube and re-incubated a total of 36 hours to reach HH16+. Fluorescent images were taken at 5x magnification to view forming trigeminal ganglia (TuJ1, green). A range of phenotypes were seen including ganglia that have mild effects on morphology (A) in comparison to the contralateral control (B) as well as severely disrupted ganglia (C and E) in comparison to their contralateral controls (D and F).

Next we performed cell counts of the Islet-1-positive cells within the ganglia and calculated averages across 7-8 serial sections in a minimum of 5 embryos, comparing against the contralateral internal control side of the embryo. In this way, we were able to measure whether or not αN-catenin knockdown in the neural crest cell population affects the placode cell number that contribute to the forming ganglia. Performing a Student's t-test on the calculated average cell numbers contributing to the experimental and contralateral control sides of the embryo indicated that there is not a significant difference in Islet-1-positive cells that contribute to the ganglia (Fig. 12L). This result indicates that abnormalities in ganglia formation upon αN-catenin knockdown are not due to alterations in the number of placode cells arriving at their final destination but likely caused by other effects, such as poor adhesion or scaffolding provided by the neural crest cells and/or aberrant signaling between the neural crest cell and placode cell population.
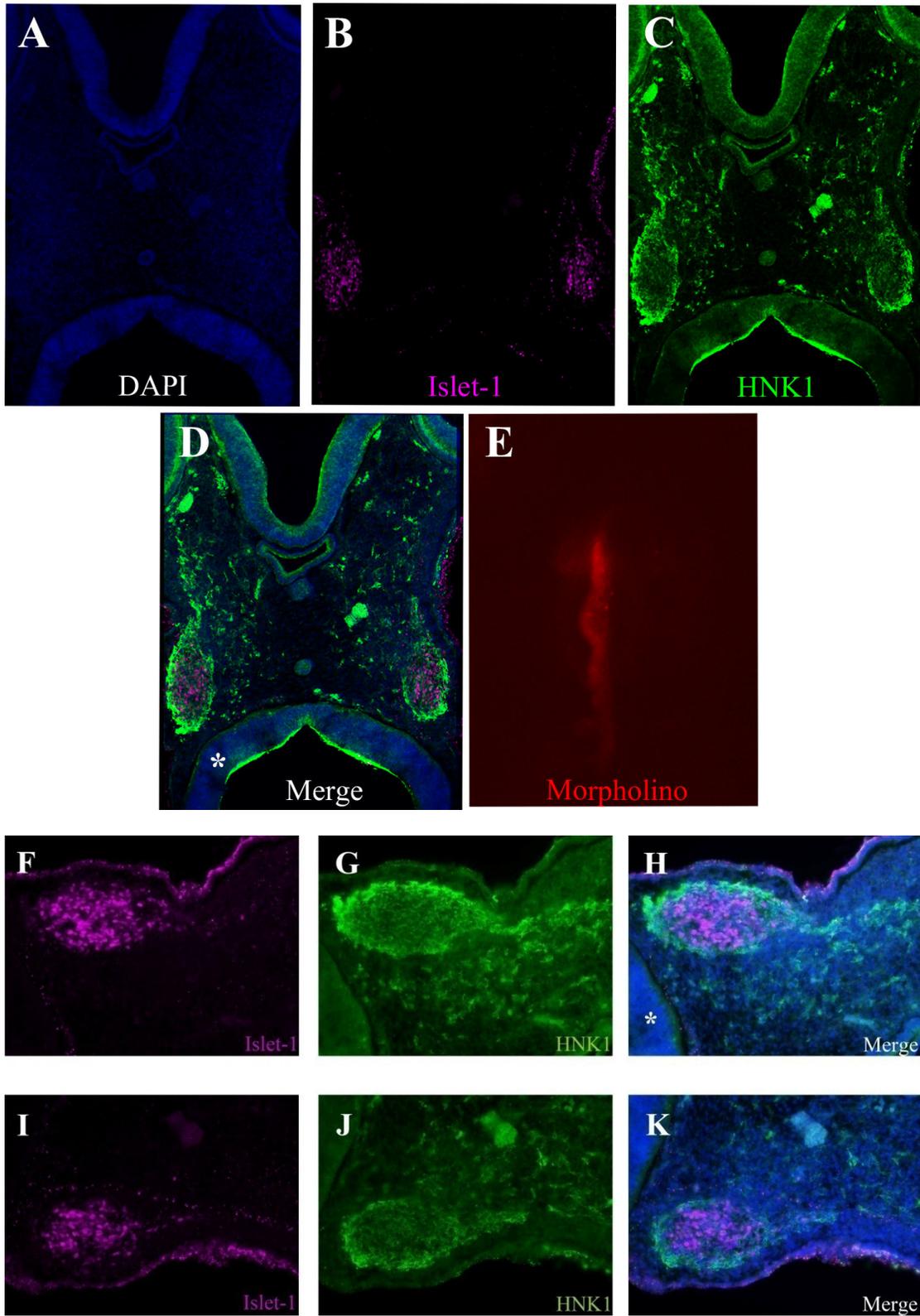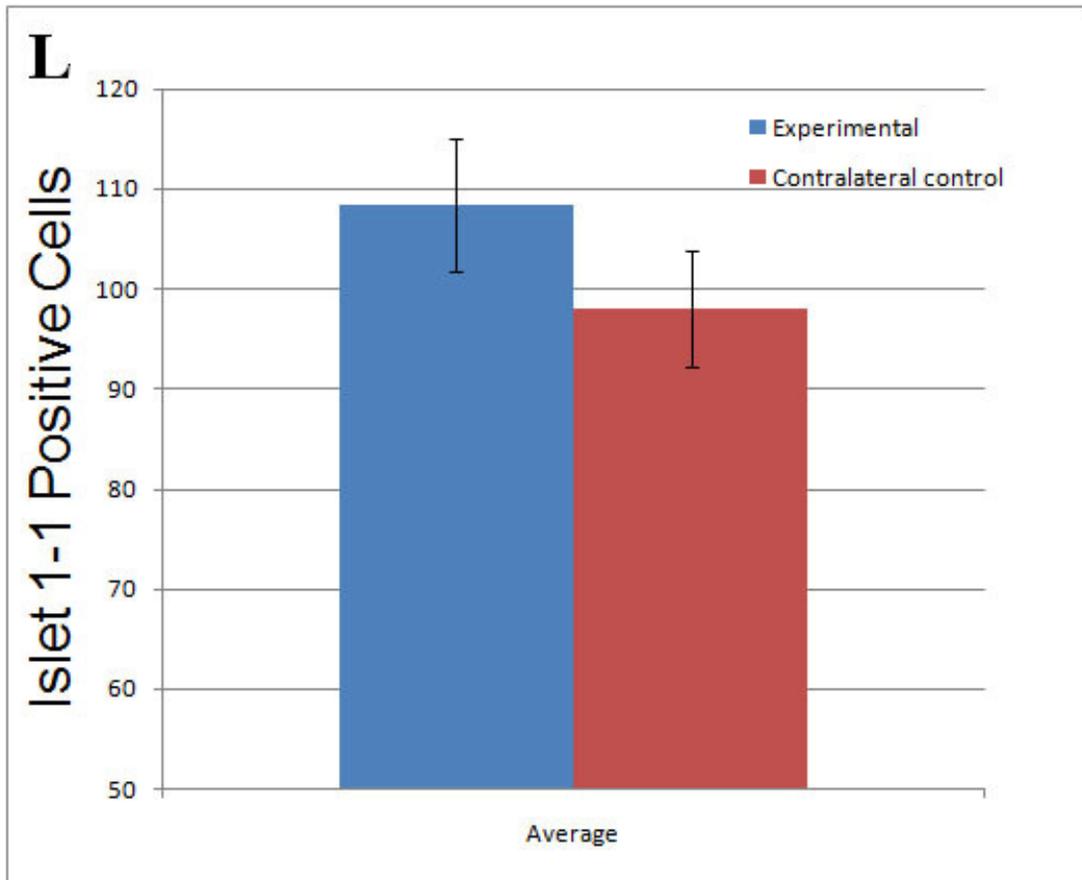
Figure 12. αN-catenin knock-down does not alter the number of placode cells contributing to the condensing trigeminal ganglia. Dual immunohistochemistry for Islet-1 (magenta) and HNK1 (green) to examine the placode and neural crest contribution to the trigeminal ganglia. (A-D) Fluorescent images taken at 10x show a section through the head of an embryo electroporated on the left side of the neural tube (E) and re-incubated a total of 38 hours to reach HH17. (F-K) The same section shown at higher magnification (20x). Individual channel images showing (A) DAPI (blue, nuclei), (C,G,J) HNK1 (green, neural crest cells),and (B,F,I) Islet-1 (magenta, placode cells). (D,H,K) Merge images show the intermixing of neural crest cells and placodal cells within the forming trigeminal ganglia. (L) Quantification showing average placode cell numbers calculated for both the treated and contralateral control sides of 5 embryos. Results are not statistically different.

4.4 αN-catenin knockdown has no long-term effect on cell death and cell proliferation in the neural tube and forming ganglia at later stages

To examine long-term effects on cell death and cell proliferation at the stages examined in this study, we performed TUNEL and Phosphohistone H3 analyses after αN-catenin knockdown on one side of the neural tube. The TUNEL assay allows us to detect apoptotic cells, and Phophohistone H3 is an antibody that marks proliferating cells. The TUNEL assay showed low numbers of apoptotic cells in both the presumptive condensing ganglia and the neural tube, and there were no appreciable changes between the treated side and the contralateral control (Fig. 13A-F). In contrast, cell proliferation was abundant throughout the embryo, especially in the apical region of the neural tube, but no differences were detected between the treated side and the contralateral control (Fig. 13G-R). These results indicate that any effects detected are not due to changes in cell death or cellular proliferation.

In general, the knockdown studies show that αN-catenin depletion enhances early neural crest migration, but does not change long-term final contributions of the neural crest or placodal cells to the trigeminal ganglia. Disruptions to the structure of the resulting ganglia, as a result, are likely due to αN-catenin depletion having effects upon normal adhesion of these two cell populations.
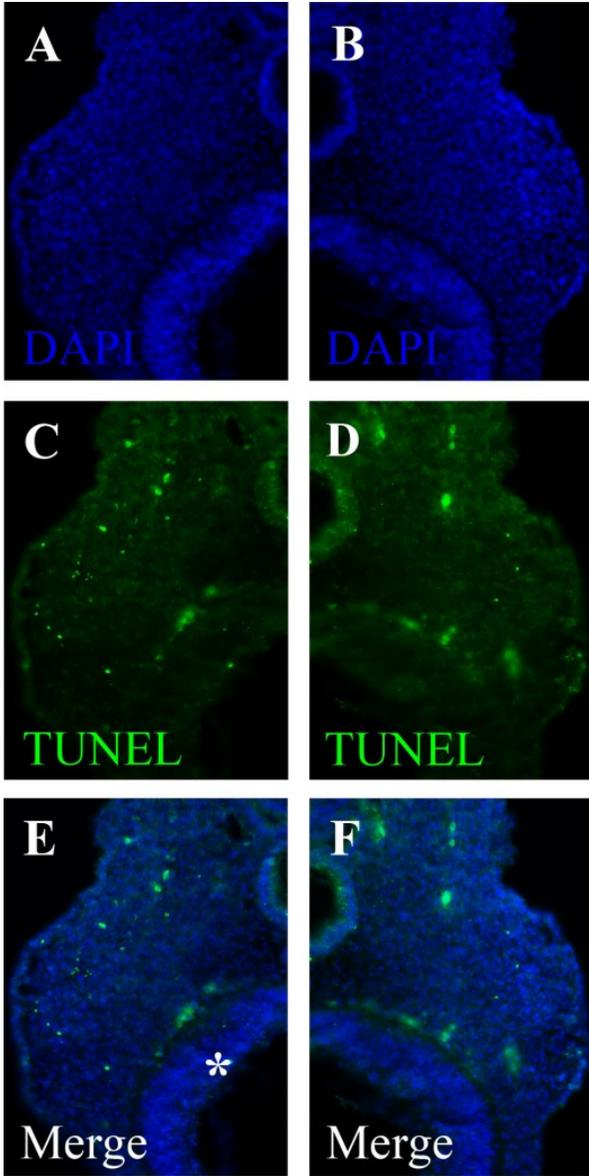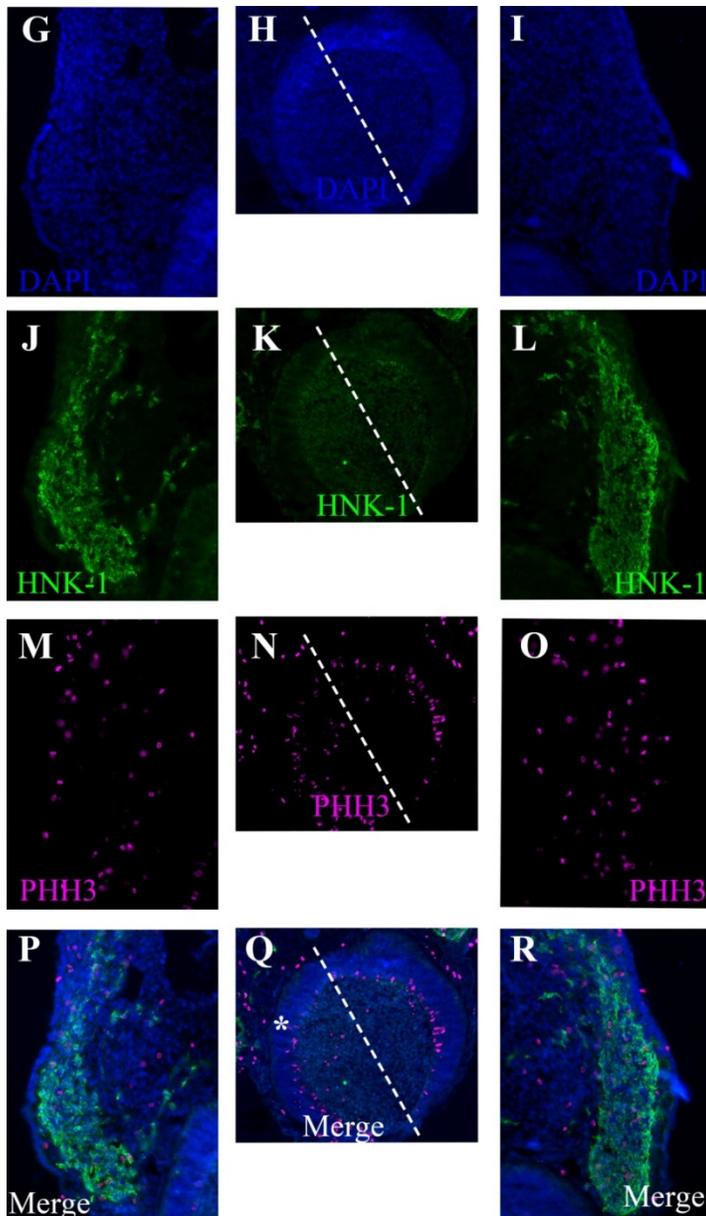
Figure 13. αN-catenin knockdown in the neural crest cells and the neural tube show no appreciable effects on cell death and proliferation. (A-F) TUNEL assay to compare treated (left) and non-treated (right) sides show little cell death occurring at these stages. (A,B) DAPI (blue, nuclei), (C,D) TUNEL fluorescein (green, apoptotic cells), (E,F) Merge image. (G-R) Immunohistochemistry to detect proliferating cells on treated (left) and non-treated (right) sides of the embryo with the neural tube shown in middle column. (G,H,I) DAPI (blue, nuclei), (J,K,L) HNK1 (green, neural crest cells), (M,N,O) Phosphohistone H3 (magenta, proliferating cells) and (P,Q,R) Merge images show proliferating cells throughout the embryo, including colocalized with neural crest cells, and on both sides of the neural tube with no detectable differences. Asterisks in (E and Q) indicate treated side of the embryos.

Chapter 5: Effects of αN-catenin overexpression in the neural crest cell population

5.1 *In ovo* imaging allows us to detect presence of GFP during development indicating successful uptake of the overexpression plasmid.

To overexpress αN-catenin in neural crest cells, αN-catenin (cloned into a GFP-expressing vector (pCIG) was injected into the chick neural tube at 2-5ss, prior to neural crest cell migration, and electric pulses were used to transfect one side of the neural tube only. Using the *in ovo* technique described in Chapter 4, we were able to detect GFP expression on the correct side of the embryo during development, indicating successful transfection with the plasmid (Fig. 14C, arrow). The embryos were then allowed to re-incubate for specific time periods and were subsequently processed to determine effects on the final localization of neural crest cells as well as the structure and placodal contribution to the condensing trigeminal ganglia.
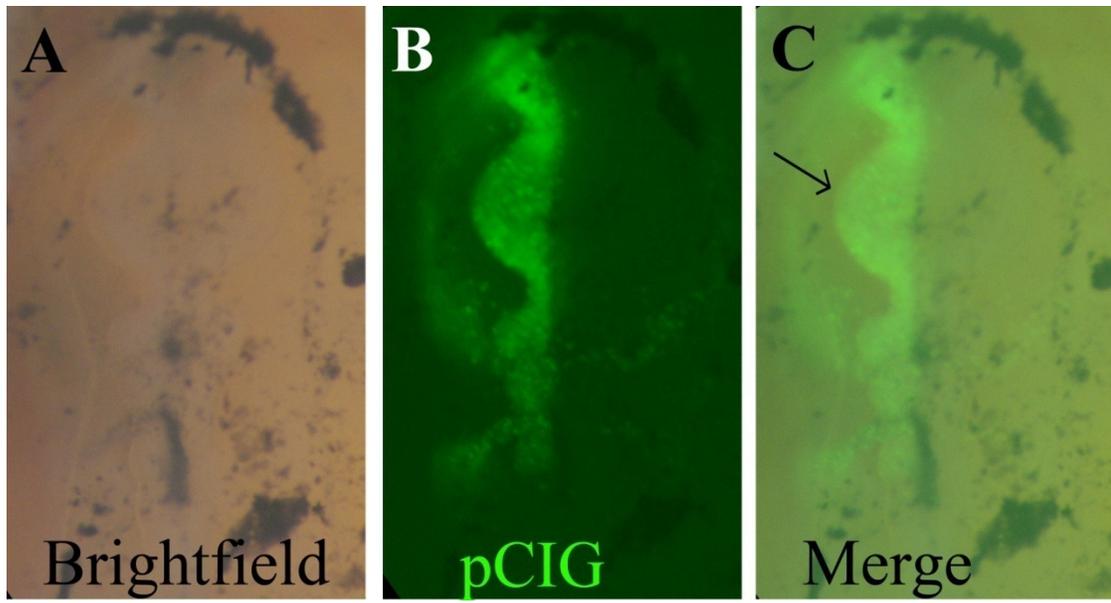
Figure 14. *In ovo* **imaging allows us to determine electroporation efficiency after transfection with a GFP-expressing plasmid.** (A) Brightfield at 40x magnification to show the outline of the neural tube of the developing embryo 15 hours post-electroporation. (B) Detection of the GFP-expressing vector shows the presence of plasmid in the embryo at this stage. (C) Merge image allows us to determine that embryos were successfully electroporated on the left side of the neural tube (arrow), and these embryos are then re-incubated an additional amount of time.

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5.2 αN-catenin overexpression decreases the neural crest cell contribution to and leads to abnormal condensation of the trigeminal ganglia.

Whole mount *in situ* hybridization was performed after electroporation on the left side of the developing embryo to target the neural crest cell population on that side predominantly. Embryos were collected at several time points to detect neural crest cell migration after αN-catenin disruption. Upon detection of *Sox10* transcripts, which mark migratory neural crest cells, the embryos were sectioned to examine the later neural crest positioning on the treated side compared against the contralateral control side (Fig. 15). Whole mount images (Fig. 15A,B) and sections (Fig. 15C-E) through the plane indicated in (Fig. 15A) show abnormal positioning and poorly condensing neural crest cells on the treated side, including detached neural crest cell aggregates (Fig.15D, arrow).
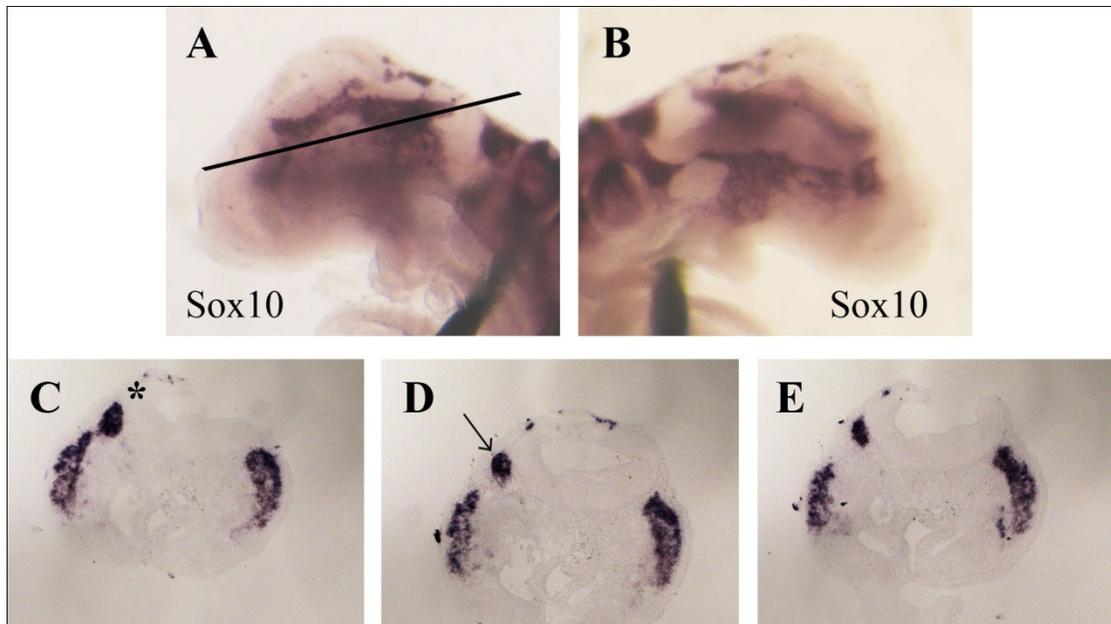
Figure 15. Detection of *Sox10* transcripts after αN-catenin overexpression on the left side shows disjointed trigeminal lobes and less condensed neural crest cells. (A,B) Brightfield images of a whole-mount embryo electroporated with αN-catenin pCIG on the left side (A) and processed to detect *Sox10* transcripts after 27 hours of incubation. (C-E) Serial sections through the head of the embryo through the migratory neural crest cell population in the plane indicated by the black bar in (A) show fewer neural crest cells on the left side and cellular aggregates (D, arrow). Asterisk in (C) indicates treated side of the embryo.

HNK1 was used as an additional marker to detect neural crest cells via protein localization using immunohistochemistry (Fig. 16). Two embryos sectioned through the trigeminal ganglia of the head at HH17 (Fig. 16A-D and E-F) show the trigeminal ganglion that is beginning to condense and form lobular structures (outlined in Fig. 16F). The condensing ganglion on the left side appears more dispersed in some sections (Fig. 16B, arrow) and cellular aggregates close to the neural tube were also detected (Fig.16D, arrowhead). Measurements to quantify the area of the condensing trigeminal lobes as outlined in (Fig. 16F) was performed in Photoshop and analyzed with a Student's t-test and found to be significantly smaller than the area of the contralateral control across 7-8 serial sections of 4 embryos (Fig.16I, $p < 0.05$).
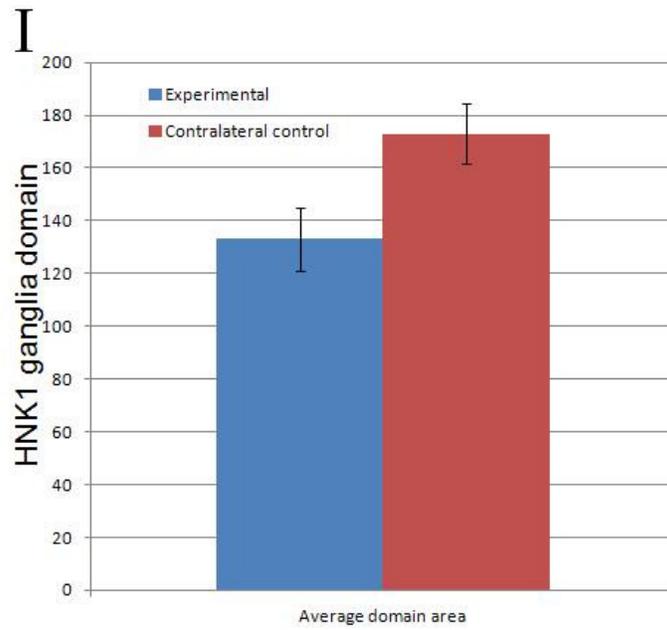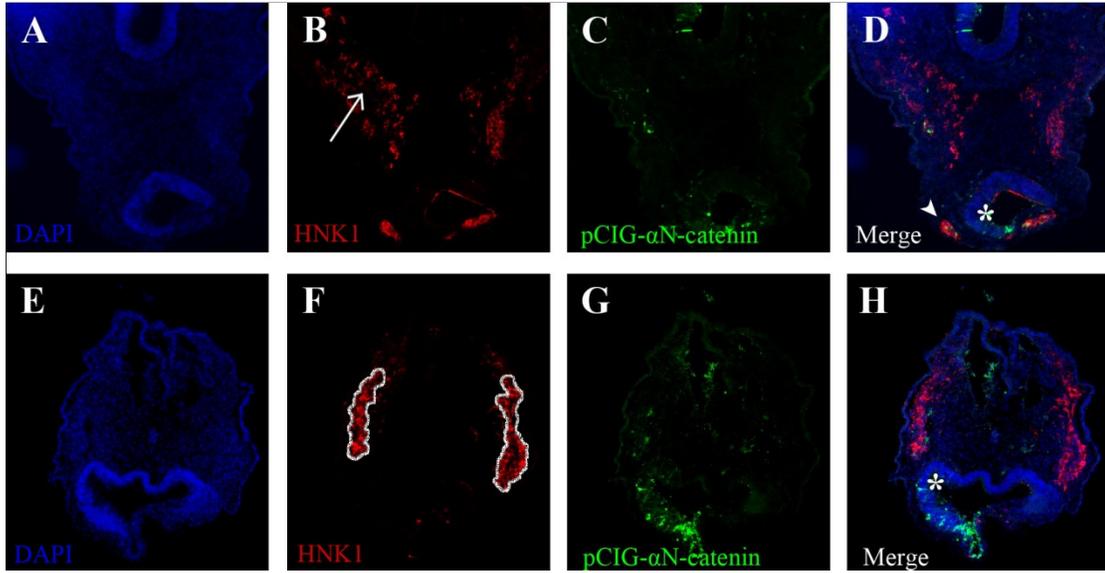
Figure 16. Detection of HNK1 protein by immunohistochemistry after αN-catenin overexpression shows decreased neural crest domain size of the condensing trigeminal ganglion. (A-D) and (E-H) are sections through the head of two separate embryos electroporated on the left side of the neural tube and re-incubated a total of 38 hours to reach HH16/17. Fluorescent images were taken at 10x magnification of DAPI to mark cell nuclei (A,E, blue) and HNK1 (B,F, red). (C and G) Detection of cells that received the αN-catenin overexpression construct was performed using an antibody against GFP. (D and G) Merge image shows the location of neural crest cells in the embryo. The trigeminal ganglia are condensing at this stage but appear more loosely dispersed on the treated side (B, arrow) and smaller in size when they do condense as shown by the outlined in (F). (I) Quantification of average area of condensing neural crest cells is shown as arbitrary square pixel units and was calculated for both the treated and contralateral control sides of 4 embryos across 7-8 serial sections. Asterisks in (D and H) indicate treated side of the embryo. Arrowhead in (D) shows cellular aggregates close to the neural tube on the treated side.

5.3 αN-catenin overexpression in the neural crest cell population leads to disrupted trigeminal ganglion structures as well as significant changes in placode contribution to the trigeminal ganglion

To determine effects of αN-catenin overexpression on the structure of the developing ganglia in whole-mount embryos, an antibody to β-neurotubulin was used to detect differentiated neurons. Using this marker and imaging the embryonic head, we can detect morphological differences on the side treated with the overexpression construct and compare them to the contralateral control (Fig. 17). Overexpression of αN-catenin in the premigratory neural crest cell population resulted in disrupted structures of the trigeminal ganglion with phenotypes ranging from mild (Fig. 17E) to severe (Fig. 17A,C). Immunohistochemical detection of GFP at these stages allows us to detect presence and location of cells that contain the overexpression construct in the proximal regions of the forming ganglion (Fig. 17G-I)

Experimental **Internal Control**

Mild

A
TuJ1

B
TuJ1

Severe

C
TuJ1

D
TuJ1

Severe

E
TuJ1

F
TuJ1

G
pCIG-αN-catenin

H
TuJ1

I
Merge
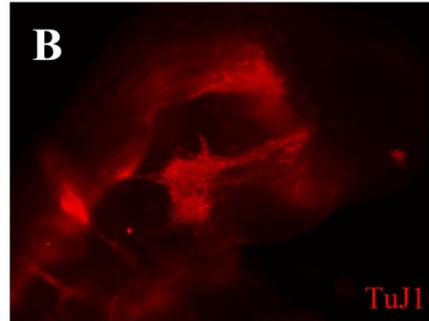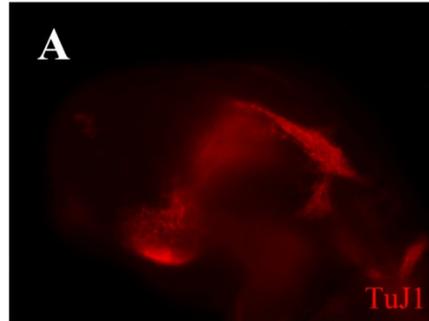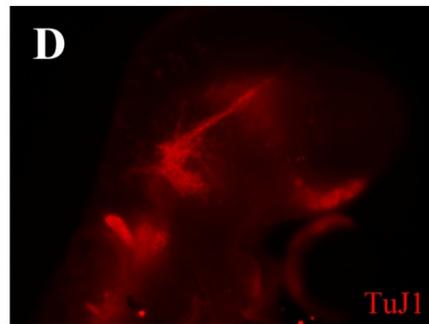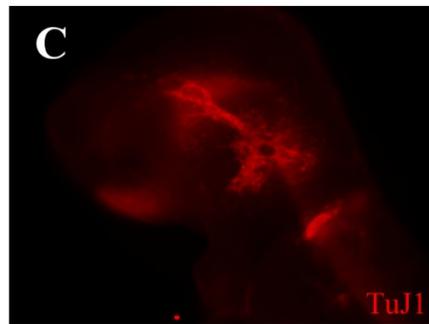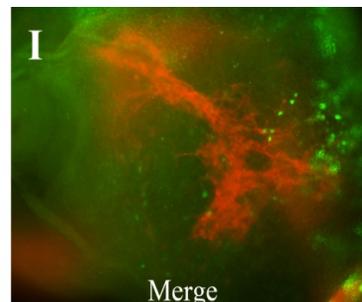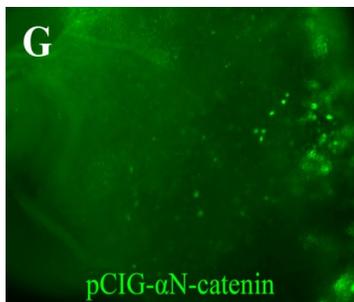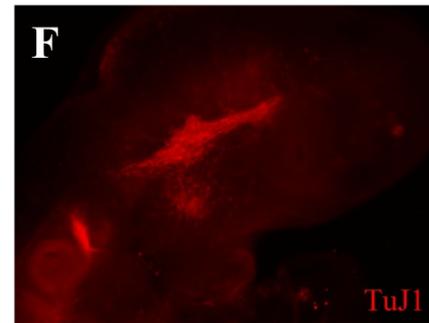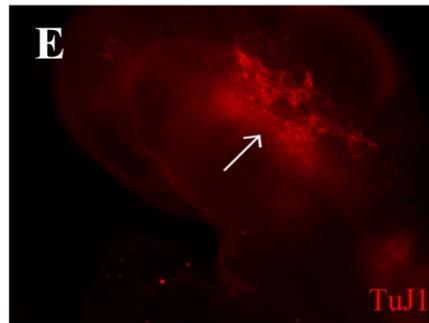
Figure 17. αN-catenin overexpression disrupts the structure of the forming trigeminal gangliaon. (A-F) Representative examples of 3 embryos electroporated on the left side of the neural tube and re-incubated a total of 36 hours to reach HH16+/17. Fluoresecent images were taken at 5x magnification to view the forming trigeminal ganglion (TuJ1, red). A range of phenotypes were seen including ganglia that appear to be somewhat smaller but have a normal structure (E) in comparison to the contralateral control (F) as well as severely disrupted ganglia (A and C) in comparison to their contralateral controls (B and D). (G-I) 10x magnification of the embryo shown in (C). (I) Merge image of GFP and TuJ1 indicating presence of the αN-catenin overexpression construct in the proximal regions of the ganglia.

To further explore the possibility that αN-catenin overexpression in the neural crest cell population has long-term effects on placode aggregation in the dual population-derived trigeminal ganglia, we used Islet-1 to mark placode cells that contribute to both lobes of the trigeminal ganglia (Fig. 18). Subsequent immunohistochemistry with HNK1 and GFP allows us to examine the complex intermixing of Islet-1-positive placodal cells with HNK1- and GFP-positive neural crest cells in the condensing ganglion in the merge image (Fig. 18D,H). Next we performed cell counts of the Islet-1-positive cells within the lobe of the ganglia and calculated averages across 7-8 serial sections in 4 embryos to compare against the contralateral internal controls. In this way, we determined that αN-catenin overexpression in the neural crest cell population leads to a decrease in the placode cell number that contribute to the forming ganglion (Fig. 18I) Performing a Student's t-test on the calculated average cell numbers contributing to the treated and contralateral control sides of the embryo indicate that there is a significant decrease in Islet-1-positive cells that contribute to the ganglion on the treated side, indicating a possible mechanism for disruption in morphology (Fig. 18I, $p < 0.05$).
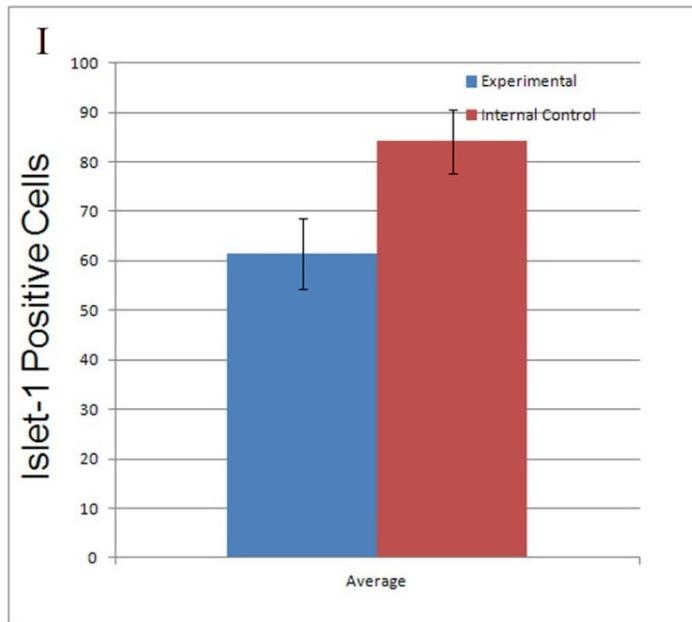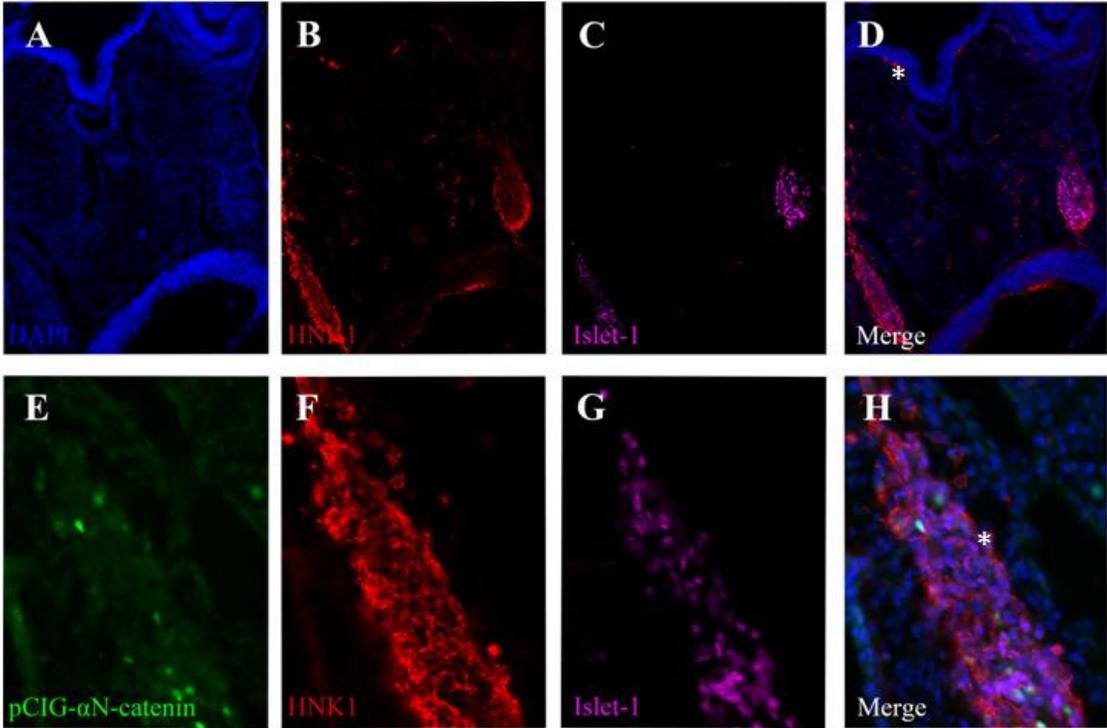
Figure 18. αN-catenin overexpression in the neural crest cells leads to fewer placode cells contributing to the condensing ganglion. Dual immunohistochemistry for Islet-1 (magenta) and HNK1 (green) to examine the placode and neural crest contribution to the trigeminal ganglia. (A-D) Fluorescent images taken at 10x show a section through the head of an embryo electroporated on the left side of the neural tube and re-incubated a total of 38 hours to reach HH17. (E-H) The same section focused on the ganglion of the treated side of the embryo shown at 20x magnification. Individual channel images showing (A) DAPI (blue, nuclei), (E) GFP (green, cells transfected with pCIG-αN-catenin), (B,F) HNK1 (red, neural crest cells), and (C,G) Islet-1 (magenta, placode cells). (D,H) Merge images show the intermixing of neural crest cells and placodal cells within the forming trigeminal ganglion. (I) Quantification showing average placode cell numbers calculated for both the treated and contralateral control sides of 4 embryos across 7-8 serial sections each shows decreased placode numbers. Asterisks in (D and H) indicate treated side of the embryo.

5.4 αN-catenin overexpression has no long-term effect on cell death and cell proliferation in the neural tube and forming ganglia at later stages

TUNEL and Phosphohistone H3 analyses were performed after αN-catenin overexpression on one side of the neural tube to examine any long-term effects on cell death and proliferation at the stages examined in this study. Similar to the knockdown studies, the TUNEL assay showed low numbers of apoptotic cells in both the presumptive condensing ganglia and the neural tube, and there were no appreciable changes between the treated side and the contralateral control (Fig. 19A-F). Cell proliferation was abundant throughout the embryo, especially in the apical region of the neural tube, but no differences were detected between the treated side and the contralateral control (Fig. 19G-R). These results indicate that the effects detected are not due to changes in cell death or cellular proliferation.

These studies show that overexpressionof αN-catenin disrupts both early and later neural crest migration leading to long-term effects on final contributions of the neural crest or placodal cells to the trigeminal ganglia. Disruptions to the structure of the resulting ganglia, as a result, are likely due to a combination of effects from poor contributions of both cell populations and the long-term effects of αN-catenin overexpression itself.
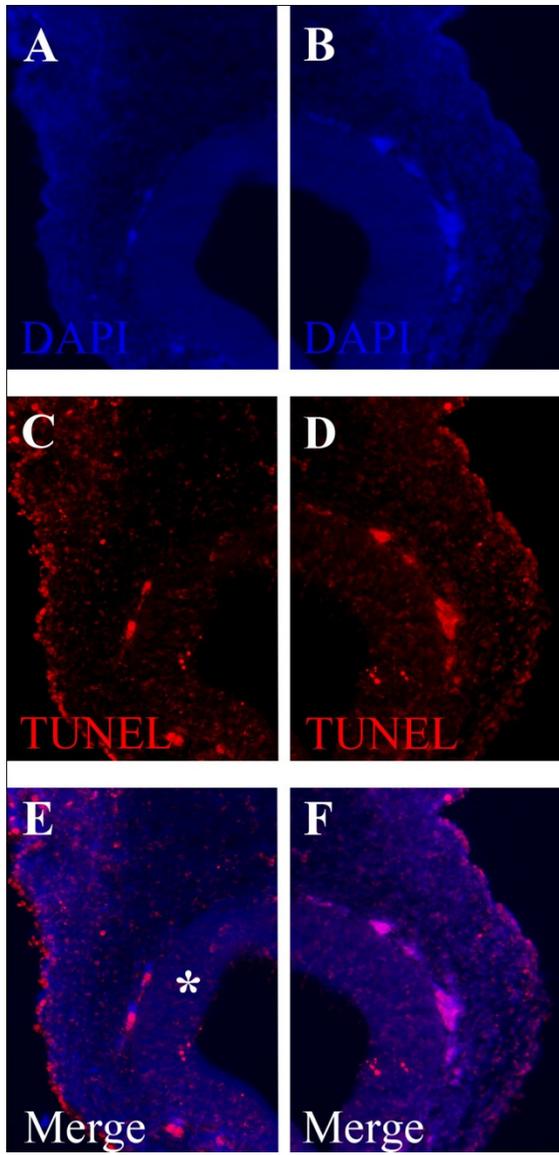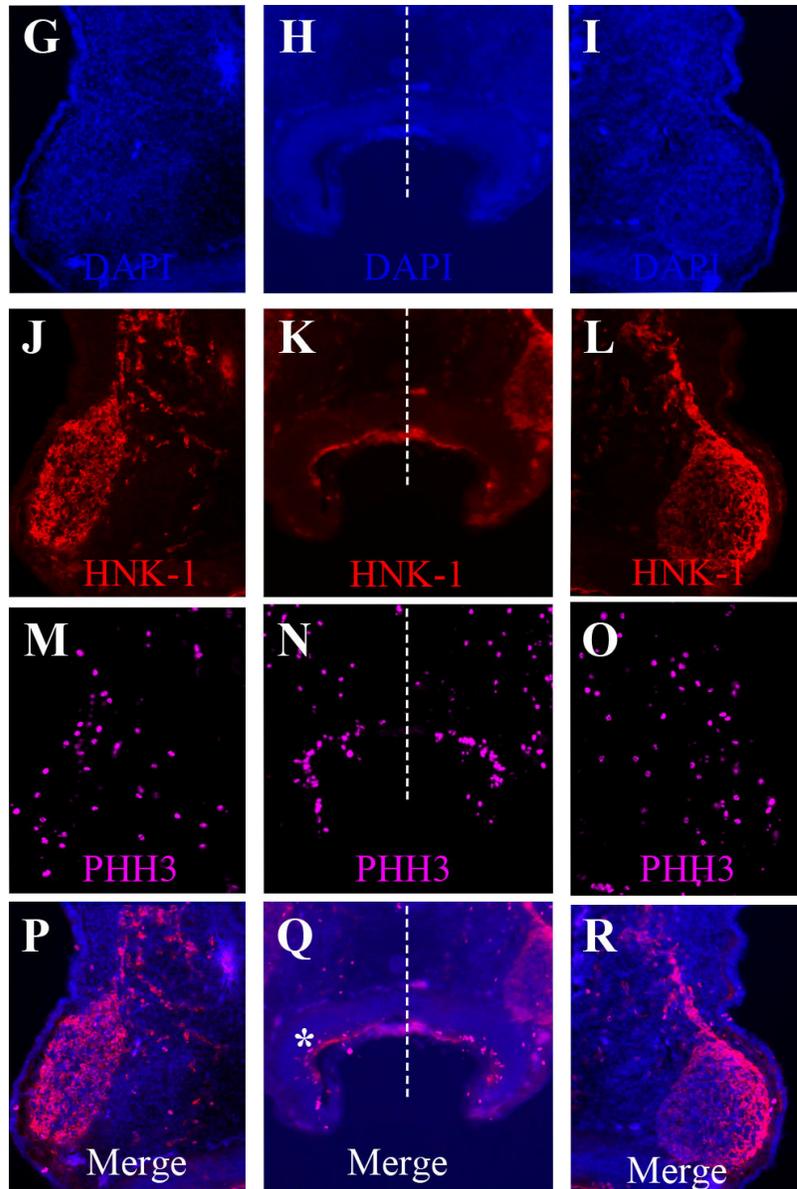
Figure 19. αN-catenin overexpression in the neural crest cells and the neural tube have no appreciable effects on cell death and proliferation. (A-F) TUNEL assay to compare treated (left) and non-treated (right) sides show little cell death occurring at these stages. (A,B) DAPI (blue, nuclei), (C,D) TUNEL TMR Red (red, apoptotic cells), (E,F) Merge image. (G-R) Immunohistochemistry to detect proliferating cells on treated (left) and non-treated (right) sides of the embryo with the neural tube shown in middle column. (G,H,I) DAPI (blue, nuclei), (J,K,L) HNK1 (red, neural crest cells), (M,N,O) Phosphohistone H3 (magenta, proliferating cells) and (P,Q,R) Merge images show proliferating cells throughout the embryo, including colocalized with neural crest cells, and on both sides of the neural tube with no detectable differences. Asterisks in (E and Q) indicate treated side of the embryos.

Chapter 6: Discussion

Neural crest cells are a highly dynamic cell population whose regulated movement and patterning have implications for the normal development of the vertebrate embryo. Regulation of molecules involved in adhesion is understood to be a crucial component in the processes of cellular delamination, migration, and coalescence, each of which are important in the transient but vital life of the neural crest cell. The regulation of the adherens junction molecule αN-catenin is known to be a mediator of initial delamination of neural crest cells from the neural tube (Jhingory et al. 2010) and has previously been shown to play roles in neural development, including axon and Purkinje cell migration (Park et al. 2002). These reasons make it a molecule of interest to examine in later stages of neural crest cell migration and differentiation, specifically in neural crest-derived neuronal development in the head. Here we show αN-catenin to be an important molecule in later stages of neural crest development and crucial for the normal formation of the trigeminal ganglia in the chick embryo.

While αN-catenin has been shown to be downregulated in early migratory neural crest cells (Jhingory et al. 2010), here we show it is re-expressed in these same cells at later stages as they condense with placode cells to form the ganglia. Previous studies examined the early effects of disrupting αN-catenin expression in the neural crest, showing that knockdown and overexpression led to enhanced and inhibited neural crest cell migration, respectively, within the first 20 hours of development post-electroporation

(Jhingory et al. 2010). To study the later effects of αN-catenin misexpression in neural crest cells, we took advantage of a convenient model system in the large and easily accessible dual-origin cranial ganglia. In this study, disrupting the expression of this adherens junction molecule in neural crest cells by blocking translation via morpholino-mediated knockdown or by overexpression has the ability to cause later neural crest-related defects associated with abnormal coalescence and/or integration of the neural crest and placodal cell populations within the trigeminal ganglia.

The morpholino-mediated knockdown, while initially causing enhanced neural crest cell migration in early stages as previously shown, appears to have no significant long-term effects on the domain size of the condensing neural crest cells measured at later stages as neural crest cells and placode cells have begun to condense. Though measured domain size was consistently larger on the treated side of the embryo, the averages of multiple serial sections of several embryos indicate that these differences were not significant. It is possible the detected effects were due to mild/moderate effects in the coalescence of the cells and not due to larger numbers of neural crest cells reaching the final destination. Measuring the domain area of HNK1, however, may not be the most efficient way to determine neural crest cell contribution, as the entire domain consists of both cell populations. Ideally we would count exact numbers of neural crest cells contributing to the condensing ganglion domain but this proves to be difficult with a cell surface marker such as HNK1. As has been shown in previous studies, early αN-catenin depletion

causes enhanced migration due to premature emigration from the neural tube

(Jhingory et al. 2010). We would expect, however, if there were a finite

number of neural crest cells destined to emigrate from the neural tube that the

untreated control side would be able to compensate over time. Alternatively,

transfected cells could die en route to their final destination and we were not

able to capture this due to fixed imaging and analysis. By the time neural crest

cells have reached their final destination when we have performed the TUNEL

assay as well as measured proliferation with phospho-histone H3 as a marker,

however, there are no appreciable differences in cell death or proliferation on

the treated side compared to the contralateral control. In this case long-term

effects, such as the abnormally structured ganglion detected with TuJ1

staining, would be due to continued αN-catenin depletion over time, and not a

change in the number of neural crest cells that reach their target.

On the other hand, the overexpression experiments did lead to smaller

neural crest-derived trigeminal ganglion domains as measured by area. This

could be a result of multiple effects, including 1) a smaller number of neural

crest cells able to delaminate from the neural tube as a result of increased

adhesion, 2) the formation of aggregates of adhered neural crest cells along

the migratory neural crest cell trajectories that are then unable to contribute to

the condensing ganglia, and/or 3) a reduction in the number of placode cells

contributing to the ganglion. Indeed, we observed a statistically significant

decrease in the number of Islet-1-positive placode cells contributing to the

trigeminal ganglion upon overexpression of αN-catenin. As a result, a range of

abnormal ganglion morphology phenotypes were detected. Additionally, no appreciable differences were noted with respect to cell proliferation and cell death on the treated side versus the contralateral control.

The differences between severity of phenotypic effects from knockdown and overexpression could be due to several factors. Both the αN-catenin morpholino and overexpression construct received by premigratory neural crest cells is continually diluted throughout various cell divisions and physical growth of the embryo during development. At later developmental stages, it is sometimes difficult to detect the presence of the morpholino, especially in individual migratory neural crest cells, which is why we rely on other methods of determining electroporation efficiency at earlier time points. Cells that have been transfected with the αN-catenin overexpression construct, however, also express GFP and are easier to detect even at the later stages by performing immunostaining for GFP to enhance the signal. This indicates that αN-catenin is still being overexpressed in those cells and still exerting effects.

The abnormal ganglion morphology that is detected upon αN-catenin perturbation is reminiscent of that observed upon ablation of the placodal or neural crest cell populations, with loss of placode and neural crest cells leading to a very disorganized ganglion and multiple small ganglion aggregates, respectively (Shiau et al. 2008). The reciprocal interaction between placode and neural crest cells is thought to involve Slit-Robo signaling. *Robo2* is expressed on ingressing placode cells, while its cognate ligand, *Slit1*, is detected on migrating neural crest cells. Misexpression of

either proteins leads to abnormal ganglion formation, similar to some of the phenotypes detected in this study (Shiau et al. 2008). Additionally N-cadherin, the neuronal sybtype of the type I classical cadherins, was found to modulate the Robo-Slit signaling in placode cells (Shiau and Bronner-Fraser 2009). Future studies could explore the interplay of neural crest cells and placode cells with respect to the established Robo2-Slit1 and N-cadherin interactions, and determine if αN-catenin is involved in these pathways. Additionally, disrupting αN-catenin expression levels by targeting the placodal cell population in the ectoderm prior to delamination and ingression would allow us to explore any potential roles for this adhesion molecule in the placode cells, which do express cadherins at the time of condensation (Shiau and Bronner-Fraser 2009).

An additional avenue for further exploration would be a closer examination of the dynamics of actin binding and/or inhibition of branching upon overexpression/knockdown of αN-catenin. α-catenins have been shown to regulate the dynamic architecture of cell-cell junctions (Drees et al. 2005, Shapiro and Weis 2010). Overexpression of αN-catenin could increase the cellular pool available, leading to dimerization and subsequent inhibition of actin branching in electroporated cells via inhibition of the Arp 2/3 complex. An additional effect of overexpression could also be the formation and stabilization of cell-cell adhesions(Gates and Peifer 2005, Drees et al. 2005). At later stages, continued overexpression in cells contributing to the ganglia would cause persistent inhibition of actin branching, thus disrupting cell-cell

dynamics among the neural crest and placodal populations, and potentially accounting for disrupted ganglion structures detected in this study. Knockdown, on the other hand, would deplete the cells of αN-catenin, allowing for disrupted cell-cell adhesion as well as functional actin branching. In earlier stages this causes enhanced delamination of neural crest cells and active migration, as observed by Jhingory et al. (2010), while at later stages, continued depletion could have deleterious effects on cell-cell interactions among neural crest and placodal populations. Both instances (overexpression or knockdown) lead to abnormal ganglion morphology at later stages through the perturbation of this junctional molecule, which then has lasting effects on the dynamics of neural crest and placode cell interactions.

An additional phenotype that was detected while performing these experiments was the disruption to the stereotypic asymmetry of the embryo that is established during the developmental stages examined. The asymmetry patterns that eventually give rise to events such as appropriate heart looping and gut coiling are established early in development prior to gastrulation, and these have long-term effects on the proper formation of the embryo (Levin 2005). Notably, embryos electroporated with the αN-catenin overexpression construct abnormally turned to the left in approximately 35-40% of the embryos examined. While it is not known if heart looping was additionally affected in these embryos, as this phenotype was not explored in depth in my thesis project, it remains an intriguing result of disrupting an adherens junction molecule. This could point to several possibilities that would be a fascinating

avenue for further research. First, as αN-catenin dimers are known to have the ability to impair actin branching via inhibition of the Arp2/3 complex(Pokutta et al. 2008, Drees et al. 2005), it remains possible that overexpression of this actin modulator in both the neural tube and neural crest cells on one side only could cause gross structural changes of the whole embryo. Second, αN-catenin has been explored as a signaling molecule in several instances and could be feeding into a signaling pathway that causes downstream effects on the left-right asymmetry route(Maiden and Hardin 2011).

We cannot rule out the possibility that results of tampering with normal αN-catenin expression levels in the neural tube and the neural crest cells stem from effects on other, less established roles of α-catenins. αN-catenin may have a possible signaling function as it was found to translocate to the nucleus with the zinc-finger protein ZASC1, leading to transcription of a putative proto-oncogene product (Bogaerts et al. 2005). αE-catenin has been implicated as a tumor suppressor and regulator of proliferation, as ablation in the mouse epidermis led to several defects including internalized cell masses, defects in polarity and hyperproliferation (Vasioukhin et al. 2001). Additionally α-catenins have been suggested as possible mediators of mechanotransduction, or the process of sensing and responding to contractile forces associated with gross morphological changes (Maiden and Hardin 2011). These alternative roles are still being explored in depth and would be a fascinating avenue for further research to study the complex and highly dynamic processes that are at work

with this molecule that may be distinct from the adherens junction, within the developing embryo.

In summary, we have shown a crucial role for αN-catenin in later migrating neural crest cells in the formation of the neural crest-derived trigeminal ganglia. Overexpression results in poor contributions from both neural crest and placodal populations and knockdown does not affect the contributions of either population, however missexpression of αN-catenin in both cases perturbs the normal structural and organizational formation of the ganglia. Our data provide further evidence that molecules associated with the actin cytoskeleton and adherens junctions, such as αN-catenin, play crucial roles in the later development of both the central and peripheral nervous systems. Taken together with our lab's prior results, these findings reveal a crucial role for αN-catenin in both early and later stages of neural crest cell formation.

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