

ABSTRACT

Title of Dissertation: **CELLULAR INTERACTIONS ORCHESTRATING TRIGEMINAL GANGLION FORMATION**

Caroline Ann Halmi, Doctor of Philosophy, 2026

Dissertation directed by: **Professor, Lisa Taneyhill, Department of Animal and Avian Sciences**

Cranial sensory ganglia are uniquely positioned within the vertebrate head to relay sensations from the environment back to the brain. The largest of these, the trigeminal ganglion, houses all of the cell bodies for the trigeminal nerve and conveys somatosensory information from a heterogeneous neuronal population derived from neural crest cells and placode cells. Although its cellular composition is well characterized, the molecular mechanisms underlying trigeminal gangliogenesis, and subsequent maturation of the trigeminal nerve, remain understudied. However, studies on cadherin proteins, a family of cell adhesion molecules, have shed light on these processes. Neural cadherin (N-cadherin) is a classical cadherin expressed in neurons and typically engages in homophilic cadherin interactions to strengthen adhesion between cells. Prior studies have revealed its importance in placode-derived neurons during their early coalescence with neural crest cells; however, additional functions for N-cadherin throughout gangliogenesis are not known. To this end, this dissertation sought to explore the role of N-cadherin throughout trigeminal neurodevelopment. Using a GFP Reconstitution Across Synaptic Partners (GRASP) assay during early ganglion assembly, we discovered that N-cadherin-expressing placode-derived neurons engage in heterophilic interactions with Cadherin-7-expressing neural crest cells to mediate initial gangliogenesis. To examine the necessity of N-cadherin across development, we used a translation-blocking morpholino to reduce N-cadherin expression in placode-derived

neurons. N-cadherin depletion produced sustained morphological defects in the ganglion and nerve branches across multiple days of development, including when neural crest cells differentiate into neurons. Building off this, we showed that N-cadherin-mediated adhesion supports axon outgrowth of both placode- and neural crest-derived trigeminal neurons, and identified a non-cell autonomous role of N-cadherin-expressing placode-derived neurons on neural crest neurite production. In our last study, we explored interactions between N-cadherin and TrkA, a receptor tyrosine kinase expressed in nociceptive neurons at later developmental stages. We characterized several post-translational modifications of TrkA, including N- and O-linked glycosylation as well as sialylation. We also discovered that N-cadherin interacts with distinct glycoforms of TrkA in a compartment-specific manner, differing between soma and axons as TrkA matures through the secretory pathway. Membrane-bound protein extractions from trigeminal ganglia further revealed multiple N-cadherin-TrkA interactions corresponding to different stages of TrkA maturation, suggesting that these interactions occur not only intracellularly but on the plasma membrane as well. Taken together, the results from this dissertation elucidate key molecular interactions underlying trigeminal gangliogenesis and provide new insights into the mechanisms orchestrating sensory neurodevelopment.

CELLULAR INTERACTIONS ORCHESTRATING TRIGEMINAL GANGLION FORMATION

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Abbreviations

BDNF	Brain-derived neurotrophic factor
CAM	Cell adhesion molecule
CHO	Chinese hamster ovary
CN	Cranial nerve
E	Embryonic day
EC	Extracellular
ECM	Extracellular matrix
EMT	Epithelial-Mesenchymal transition
GFP	Green fluorescent protein
GM130	Golgi matrix protein 130
GRASP	GFP reconstitution across synaptic partners
HH	Hamburger-Hamilton
miR	Micro-RNA
MM	Mismatch morpholino
MmV	Maxillomandibular
MnV	Mandibular
MO	Morpholino
MTs	Microtubules
MxV	Maxillary
N-cadherin	Neural-cadherin
NGF	Nerve growth factor
NT-3	Neurotrophin-3
NT-4/5	Neurotrophin-4/5
OpV	Ophthalmic
PFA	Paraformaldehyde
PHH3	Phospho-Histone H3
PLA	Proximity ligation assay
RTK	Receptor tyrosine kinase
SERCA2	Sarco/Endoplasmic Reticulum Calcium ATPase 2
srGAPs	Slit-Robo GTPase-activating proteins
ss	Somite stage
TKD	Tyrosine kinase domain
Trk	Tropomyosin receptor kinase
Tubb3	Tubulin Beta 3 Class III

Chapter 1: Literature Review

1.1 Overview of cranial ganglia and cell populations

The peripheral nervous system plays a critical role in allowing organisms to detect and respond to environmental cues. To this end, neurons of the peripheral nervous system transmit sensory information from the body back to the brain. A portion of these neurons condense into sensory ganglia, and specifically within the head, are known as cranial ganglia. While some of the cranial nerves function to transmit only sensory information or motor information, a subset of them conveys both. Of the five cranial nerves (trigeminal, facial, glossopharyngeal, vagus, and spinal accessory), the paired trigeminal nerves are unique in that they are the largest of all the cranial nerves and extensively innervate many facial regions. The trigeminal ganglion is formed through complex cellular and molecular interactions and relies on communication between neural crest cells that delaminate and migrate from the dorsal neural tube and placode cells that ingress from the ectoderm. When these two cell populations coalesce, the ganglion is formed, with neurons generated from both neural crest and placode cells, while supporting glia arise from only the neural crest. In order for this structure to properly form, trigeminal neurons must develop and specialize properly, which relies on several coordinated processes during embryonic development. This review will serve to explore the cells and molecules that underlie trigeminal gangliogenesis.

1.1.1 Neural Crest Cells

Neural crest cells are a unique group of cells that are associated with the development of vertebrates and generate diverse cell types including craniofacial cartilage and bone, melanocytes, neurons and glia of the peripheral nervous system, and cardiac and enteric structures (Le Douarin & Kalcheim, 1999; Schock et al., 2023; Simoes-Costa & Bronner, 2015). This remarkable multipotency has led to their description as the “fourth germ layer” (Sommer, 2010). Derived from

the embryonic ectoderm, neural crest cells originate from the neural folds at the neural plate border (Basch et al., 2006) (Fig. 1.1). The unique mode of neural crest specification has been of interest for decades, and now with the advances of single-cell and multi-omics technologies, we are beginning to better understand the regulatory elements underlying their versatility. Contrary to earlier chick-based studies suggesting that neural crest fate is acquired during gastrulation (Basch et al., 2006), more recent work suggests that neural crest cells only acquire distinct transcriptional profiles during neurulation (R. M. Williams et al., 2022). However, this interpretation is species-specific and remains somewhat controversial as it relies primarily on transcriptomic analyses without taking into account other factors such as the proteome or functional experiments. Other chick studies discovered that Yamanaka factors Oct4 and Sox2 bind to neural crest-specific enhancers to promote their specification (Hovland et al., 2022), and transcriptomic analyses of the ectoderm revealed that a pluripotency-like signature is maintained throughout neurulation but later becomes restricted to neural crest cells, offering insight into how these cells are able to differentiate into their numerous derivatives (Pajanoja et al., 2023). While these findings provide information about how neural crest cells maintain stem cell-like characteristics, they lack *in vivo* functional validation, which would provide stronger evidence surrounding neural crest cell specification.

In their premigratory stage, neural crest cells are pseudo-epithelial due to the presence of specific junctional complexes that confer adhesion amongst them (Taneyhill & Schiffmacher, 2017). During neurulation, neural crest cells become motile by undergoing an epithelial-to-mesenchymal transition (EMT) and then migrate away from the dorsal neural folds to their destined locations (Taneyhill, 2008). This process involves a series of events that changes epithelial cells into mesenchymal cells, including the loss of high affinity adherens junctions and apicobasal polarity, the acquisition of motility, and penetration of the basement membrane, the

latter of which is often an active process involving degradation of basement membrane proteins (Ahlstrom & Erickson, 2009). In addition, changes in cell shape require the reorganization of the plasma membrane, which is in part mediated by lipid-modifying enzymes, as perturbations of these enzymes in chick results in decreased neural crest cell migration (Piacentino et al., 2022). Experiments performed in frog, zebrafish, and avian embryos discovered that induction of EMT is regulated through a series of different signaling events including pathways such as BMP, Notch/Delta, FGF, and Wnt (Coffman et al., 1993; Cornell & Eisen, 2002; Endo et al., 2002; Kerosuo & Bronner-Fraser, 2012). Interestingly, mammalian neural crest cells have recently been shown to become migratory not just through EMT, but also through cell extrusion. This involves detachment of neuroepithelial cells due to increased cell density and pressure, indicating that a subset of neural crest cells delaminate via different mechanisms (Moore Zajic et al., 2025). Disruptions in the migration, proliferation, and/or differentiation of neural crest cells can lead to a diseases commonly called neurocristopathies (Etchevers et al., 2006), which manifest as craniofacial malformations, loss of pigment in the skin, as well as different cancers (Watt & Trainor, 2014). Due to the broad range of neurocristopathies, it is critical to understand neural crest cell development to develop treatments and cures for these conditions.

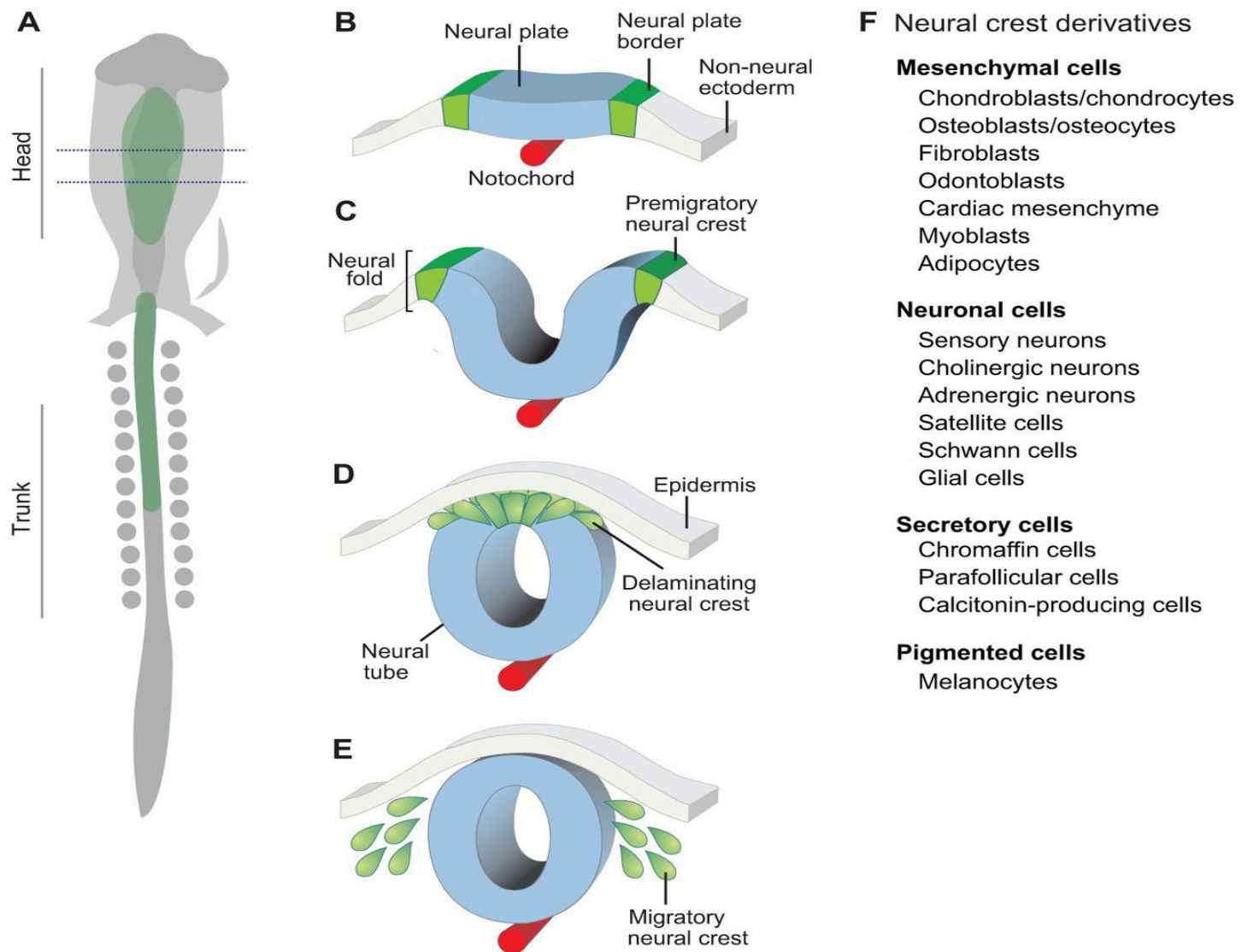


Fig. 1.1 The neural crest is a multipotent cell population.

(A) Dorsal view of a chicken embryo, with the neural crest cell population colored green. (B-E) Progression of the invagination of the neural plate during neurulation, followed by neural crest cell delamination and migration. (F) Derivatives of neural crest cells. Adapted from (Simões-Costa and Bronner, 2015).

1.1.2 Placode cells

During embryogenesis, ectodermal thickenings in the cranial region of vertebrates give rise to neurogenic placodes, which contribute neurons to many cranial sensory ganglia. These areas of thickenings develop through apicobasal elongation of cuboidal cells in the inner layer of the head ectoderm (Fig. 1.2) (Park & Saint-Jeannet, 2010). While they share some behavioral similarities to neural crest cells, namely, the capacity to delaminate and migrate, placode cells do not do so through EMT (Graham et al., 2007). Instead, they typically differentiate into neurons immediately upon their delamination from the surface ectoderm. Moreover, unlike the neural crest, placode cells do not express proteins typically associated with EMT, and migrate as streams of differentiating neurons.

While less is known about the signaling pathways that regulate placode formation and delamination, studies in *Xenopus* and mouse have identified a small group of transcriptional regulators critical for pre-placodal ectoderm specification. Loss-of-function experiments demonstrated that *Eya1* and *Six1* are both required for placodal fate, as their absence reduces the number of placodal neurons, as indicated by lower expression of *Tubb3* (class II β -tubulin) and *NeuroD* (Ikeda et al., 2007; Schlosser et al., 2008; Zou et al., 2004). More recently, the transcription factor *Foxd4*, originally characterized in *Xenopus* neuroectoderm development, was shown to maintain neural progenitor identity and promote the transition from pluripotency to neurogenesis in mammalian stem cells (Sherman et al., 2017). How placode cells acquire their fate has further been advanced by RNA- and ATAC-sequencing. Analysis of the chick neural plate border revealed that while these cells are initially heterogeneous and co-express transcription factors associated with different developmental fates, they eventually become committed to the placodal fate (Hamrud et al., 2025; R. M. Williams et al., 2022). In addition, distinct pre-placodal

ectoderm markers were detected after sequencing from inner ear organoids, mirroring those observed during *in vivo* development and shedding light on how placodal cells are generated in culture (Steinhart et al., 2023).

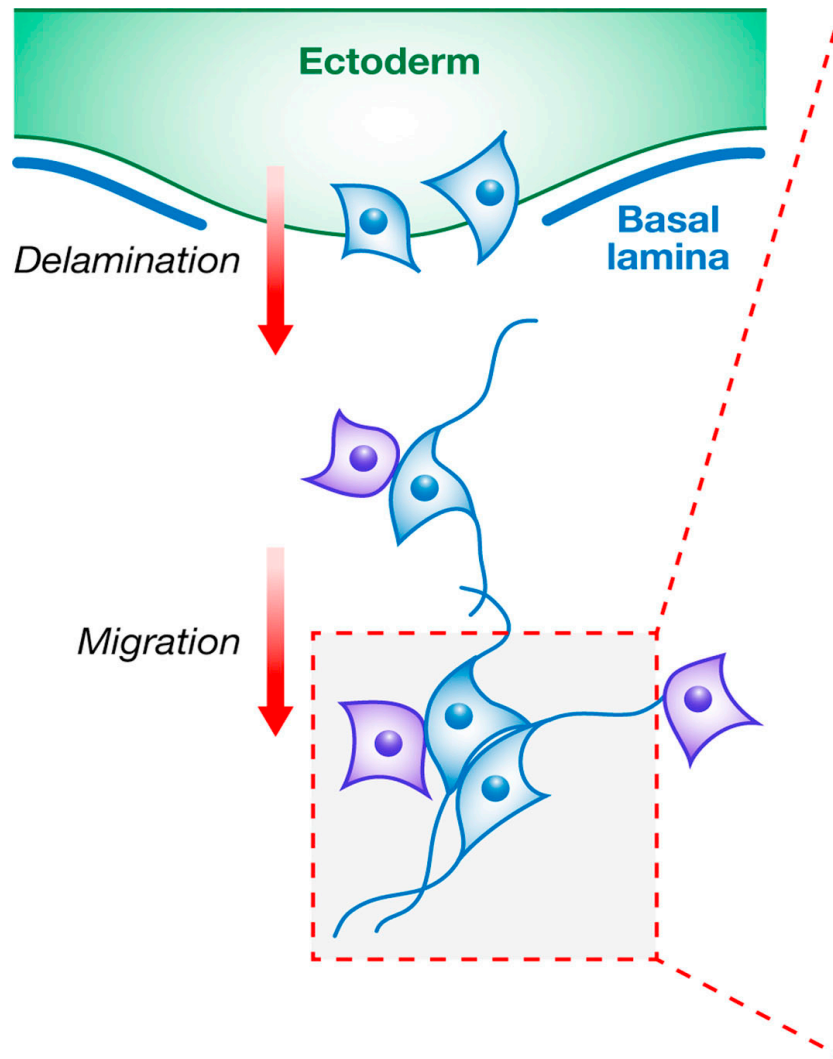


Fig. 1.2 Delamination and migration of placode cell-derived neurons.

Placode cell-derived neurons or neuroblasts migrating inwards from the ectoderm, interacting with neural crest cells during the formation of cranial ganglia. Adapted from (Breau and Schneider-Maunoury, 2015).

Cranial placodes have the ability to rearrange as epithelial cells and move as cohesive clusters (Breau & Schneider-Maunoury, 2015). They originate at distinct positions along the anterior-posterior axis and are named accordingly (e.g., adenohipophyseal placode, olfactory placode, lens placode, trigeminal placode, profundal placode, epibranchial placode, and the otic placode) (Schlosser, 2006)). Of these, the lens and adenohipophyseal placodes are non-neurogenic, producing the lens vesicle and the anterior lobe of the pituitary gland, respectively. The remaining placodes are neurogenic, as they differentiate into sensory neurons of the organs and ganglia of the developing vertebrate. While each placode functions exclusively in its respective location to generate sensory structures and will be discussed in brief, the trigeminal ganglion will be explored in greater detail later. The olfactory placode generates epithelial and secretory cells of olfactory organs, and uniquely, glial cells (Norgren et al., 1992). The otic placode forms the inner ear and sensory neurons of the vestibulocochlear ganglion. The epibranchial placodes contribute to the facial, glossopharyngeal, and vagal nerves. Lastly, the profundal and trigeminal placodes generate sensory neurons that interact with neural crest cells to form the profundal and trigeminal nerve, respectively (Schlosser, 2006).

Cranial placodes initially overlap but become regionally distinct as development progresses (Bellairs & Osmond, 2014). The trigeminal placode, which consists of the ophthalmic and maxillomandibular placodes, gives rise to all the placode-derived neurons of the trigeminal ganglion. Despite contributing neurons to the same ganglion, these placodes differ in developmental timing and fate. The ophthalmic placode produces primarily post-mitotic neurons that delaminate and migrate as neurons, whereas the maxillomandibular placode generates neuroblasts that complete differentiation after reaching the trigeminal ganglion (Begbie, 2002; Graham & Shimeld, 2013; McCabe et al., 2009; Shiao et al., 2011). Given their close spatial and

temporal interactions, perturbations in placode formation can impact neural crest development, contributing to congenital sensory defects including blindness, deafness, and anosmia (Dincer et al., 2013; Moody & LaMantia, 2015; Ranganathan et al., 2025).

1.1.3 Trigeminal Ganglion

The trigeminal (Vth) ganglion is composed of neurons derived from both neural crest cell and placode cell populations. In chick, the trigeminal placode contributes primarily to the distal portion of the ganglion, while neural crest cells form the proximal portion and associated glia (Fig. 1.3) (Steventon et al., 2014). This organization appears to be species specific, as studies in mouse could not reliably tell the cellular origin of a trigeminal neuron based on location alone. Instead, placode- and neural crest-derived neurons are intermingled in a more mosaic pattern (Karpinski et al., 2016, 2022; Leonard et al., 2022). The trigeminal ganglion houses the cell bodies for the trigeminal nerve (V), the largest of the cranial nerves, which mediates sensations of pain, touch, and temperature in the face. Due to the large facial area this nerve innervates, disruptions in trigeminal ganglion development can have long-standing impacts on sensory function. When trigeminal neurons are activated, they stimulate bordering glia cells, initiating inflammatory signaling that leads to subsequent facial pain (Thalakoti et al., 2007). Conversely, impaired function of the ganglion can lead to sensory deficits. Three of the most common maladies linked to trigeminal nerve dysfunction are Waalenburg syndrome, cluster headaches, and trigeminal neuralgia. Waalenburg syndrome occurs due to stroke and results in ipsilateral sensory loss in the trigeminal nerve, whereas cluster headaches present as severe unilateral facial pain headaches, with an unknown cause. Trigeminal neuralgia is typically caused by mechanical compression on the nerve due to aneurysm, tumor, or trauma, and can often be alleviated with surgery (Huff & Daly, 2020).

The dual origin of the trigeminal ganglion was established over 60 years ago using the chick embryo (Hamburger, 1961a). Isotope staining and tissue transplantation experiments defined the developmental timeline of when neural crest- and placode cell- populations coalesce to form the ganglion (D'Amico-Martel & Noden, 1980, 1983). Like neural crest cells, placodal neurons are also capable of collective migration (Breau & Schneider-Maunoury, 2015; Theveneau et al., 2013). Although the molecular mechanisms mediating neural crest-placode cell interactions remain obscure, current data indicate that neural crest cells provide corridors that guide placodal neurons to their proper locations, acting as a more permissive substrate than the surrounding mesoderm (Freter et al., 2013a). Ablation studies further reveal the dependence of these two cell types on one another. While removal of neural crest cells causes placodal neurons to become disorganized, placode cell ablation prevents neural crest cells from condensing properly (Shiau et al., 2008). Furthermore, neural crest and placode cells exhibit “chase and run” behavior, where neural crest cells promote the migration of placode cells to their terminal destinations (Theveneau et al., 2013). Most recently it was discovered that microRNA (miR)-203, which is initially required for neural crest cell migration, is reactivated in neural crest cells as they coalesce with placode cells during trigeminal ganglion formation. Secreted from neural crest cells, miR-203 is taken up by placode cells and is needed to regulate gangliogenesis, as perturbations to miR-203 result in aberrant coalescence and ganglion size (Bernardi et al., 2024). These reports, in addition to other studies, indicate an intricate dynamic between neural crest cells and placode cells that point to important reciprocal interactions (Hamburger, 1961a; Moody & Heaton, 1983; Shiau et al., 2008a). Although much has been understood about the influence of neural crest cells on surrounding cell types, placode cells included, there is still limited knowledge about how placodal neurons influence the migration and differentiation of neural crest cells.

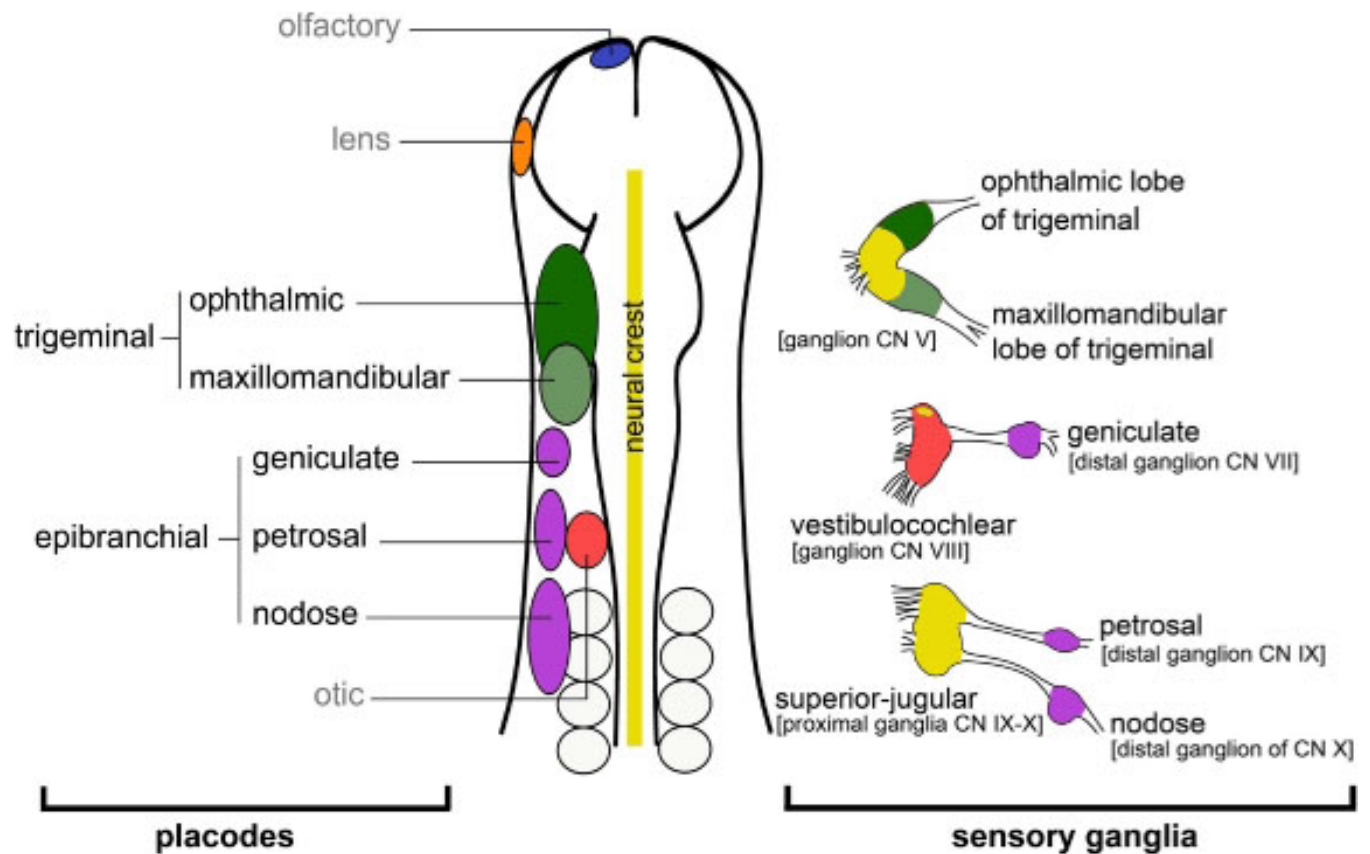


Fig. 1.3 Trigeminal and epibranchial placode contribution to sensory ganglia in the vertebrate embryo.

Color-coded diagram depicting contributions of trigeminal and epibranchial placodes, and neural crest cells to the formation of cranial nerve (CN) V, VII, IX, and X. Adapted from (Park and Saint-Jeannet, 2010).

1.1.4 Pioneer Neurons

In neuronal systems, the earliest populations of neurons are called the pioneer neurons, as they migrate to targets first and provide a scaffold for later born neurons, termed follower neurons, to grow along (Bate, 1976). Although both pioneers and followers will contribute to one nerve bundle, a tightly organized grouping of axons like those seen in the trigeminal nerve, these two cell populations have different behaviors and morphologies. Pioneer neurons tend to exhibit greater growth cone complexity and display different kinetics (Bak & Fraser, 2003; Kulkarni et al., 2007) than followers, since they must interact directly with the extracellular matrix (ECM) and interpret environmental cues to establish their proper trajectories. Followers, on the other hand, have a more straightforward role, primarily fasciculating to pioneer neurons to stabilize and enhance the emerging nerve bundle, a process that relies heavily on cell adhesion molecules (Breau & Trembleau, 2023). Most recently, single-cell RNA-sequencing of zebrafish lateral lines discovered that these two populations are transcriptionally distinct, making it the first report to establish molecular differences between the two (Woodruff et al., 2025).

While there is some understanding of the function of pioneer neurons within vertebrates (Eisen et al., 1986; Ghosh et al., 1990; Gong & Shipley, 1995), much of our knowledge in this area has come from studies in invertebrates (Clarke et al., 2020; Hidalgo & Brand, 1997; Klose & Bentley, 1989; D. M. Lin et al., 1995). Still, there is conflicting data about whether pioneer neurons are necessary and unique cells, and additionally, their importance appears to be system-dependent. Within the trigeminal ganglion, there is very limited information surrounding the function and relevance of pioneer neurons. One study in mouse found the earliest trigeminal neurons of the ophthalmic lobe reaching their targets by embryonic day (E) 9.5 (Stainier & Gilbert, 1990). Within the chick, the use of a monoclonal antibody against Tubb3 has aided in understanding that placode

cells express Tubb3 before they leave the ectoderm, while neural crest cells express Tubb3 later, after they differentiate into neurons (Moody et al., 1989). This is in keeping with findings on the timeline of trigeminal sensory neuron differentiation in the chick, with placode-derived neurons becoming post-mitotic and differentiating within 2-5 days, while neural crest-derived neurons do not do so until 4-7 days of incubation (D'Amico-Martel & Noden, 1983). During this time, placodal neurons begin to grow towards the periphery to make their initial connections with their targets. This cumulative knowledge suggests that placodal neurons, or a subset of them, are pioneers that serve as templates for the later differentiating neural crest-derived neurons to grow and extend axons (Moody et al., 1989); however, this has never been definitively proved. Furthermore, molecules orchestrating this process remain unknown.

1.2 Signaling pathways and molecules mediating gangliogenesis

1.2.1 Signaling pathways

While much effort has been made to understand the cellular and molecular mechanisms underlying trigeminal ganglion development, the upstream signaling pathways that coordinate these events in a spatiotemporal manner remain relatively obscure. Even so, a few key signaling pathways have been identified. Hox genes are critical transcription factors that establish the anterior-posterior axis of the body plan. In cranial development, they are expressed in the hindbrain, and thus impact the positional identity and migratory streams of neural crest cells arising therein. Retinoic acid acts upstream of Hox genes and is capable of transforming the identity of rhombomere specific-derived structures such as bone, cartilage, and cranial nerves (Afzal et al., 2023; Gavalas et al., 2003; Marshall et al., 1992; Trainor et al., 2002). Canonical Wnt and FGF signaling are both needed for ophthalmic and maxillomandibular placode delamination and

differentiation. Wnt signaling induces the expression of Pax3, the earliest transcription factor expressed in ophthalmic placode cells (Stark et al., 1997a), and inhibition of Wnt leads to the inability of these cells to acquire an ophthalmic placodal fate (Canning et al., 2008; Lassiter et al., 2007). Further examination of Wnt signaling in these placodes led to the identification that disrupting the FGF receptor, Fgf8, caused premature differentiation of both ophthalmic and maxillomandibular placodes by inducing Pax3 expression earlier, identifying two interactive pathways during trigeminal placode cell specification (Canning et al., 2008).

Once cells reach their terminal destinations, they rely on signals from their surrounding environment to assemble and form the trigeminal ganglion. Wnt signaling, and more specifically Wise, a Wnt modulator expressed in the surface ectoderm, is necessary during this process. Overexpression of Wise is sufficient to ectopically form ganglion-like structures that contain both neural crest and placode cells. Given that Wise is expressed in the ectoderm, these data suggest that it acts non-cell autonomously on neural crest cells to regulate trigeminal ganglion assembly (Shigetani et al., 2008a). Additionally, semaphorin/neuropilin signaling is critical for trigeminal condensation and axon outgrowth. Studies in both mouse and chick have demonstrated that perturbations to this signaling pathway lead to incorrect neural crest migratory patterns, resulting in the typically observed well defined neural crest streams becoming unorganized and dysregulated (Gammill et al., 2007; Schwarz et al., 2008). Further, mice lacking the Semaphorin III gene experience neuronal abnormalities including defasciculation of axons, further supporting the role of this signaling pathway in axon guidance (Ulupinar et al., 1999).

Lastly, the Slit/Robo pathway has also been identified as a modulator of gangliogenesis (Fig. 1.4). Slit1, expressed in neural crest cells, and its receptor Robo2, expressed in placode-derived neurons, are both crucial for proper ganglion assembly. Perturbations to either

recapitulates disorganized cellular phenotypes observed in neural crest or placode cellular ablation studies, indicating that Slit/Robo signaling acts as a positive regulator of placode delamination, cell position, and axon guidance (Shiau et al., 2008). Although Slit/Robo signaling is classically associated with repulsive guidance, in the forming chick trigeminal ganglion, it serves as an attractant, drawing placode-derived neurons to neural crest cells. In line with this, Slit-Robo GTPase activating proteins (srGAPs), a family of Rho GTPases, mediate the downstream cytoskeletal responses to this attractive signaling (Wong et al., 2001). Studies in mouse have suggested that srGAPs regulate neural migration and axon branching, control actin polymerization in the neuronal cytoskeleton, and promote migration of commissural neurons during midline crossing (Bacon et al., 2009). Most recently, bulk RNA-sequencing of forming trigeminal ganglia taken from chick embryos was performed, serving as a valuable resource to identify potentially unknown regulators of gangliogenesis (Leonard et al., 2025).

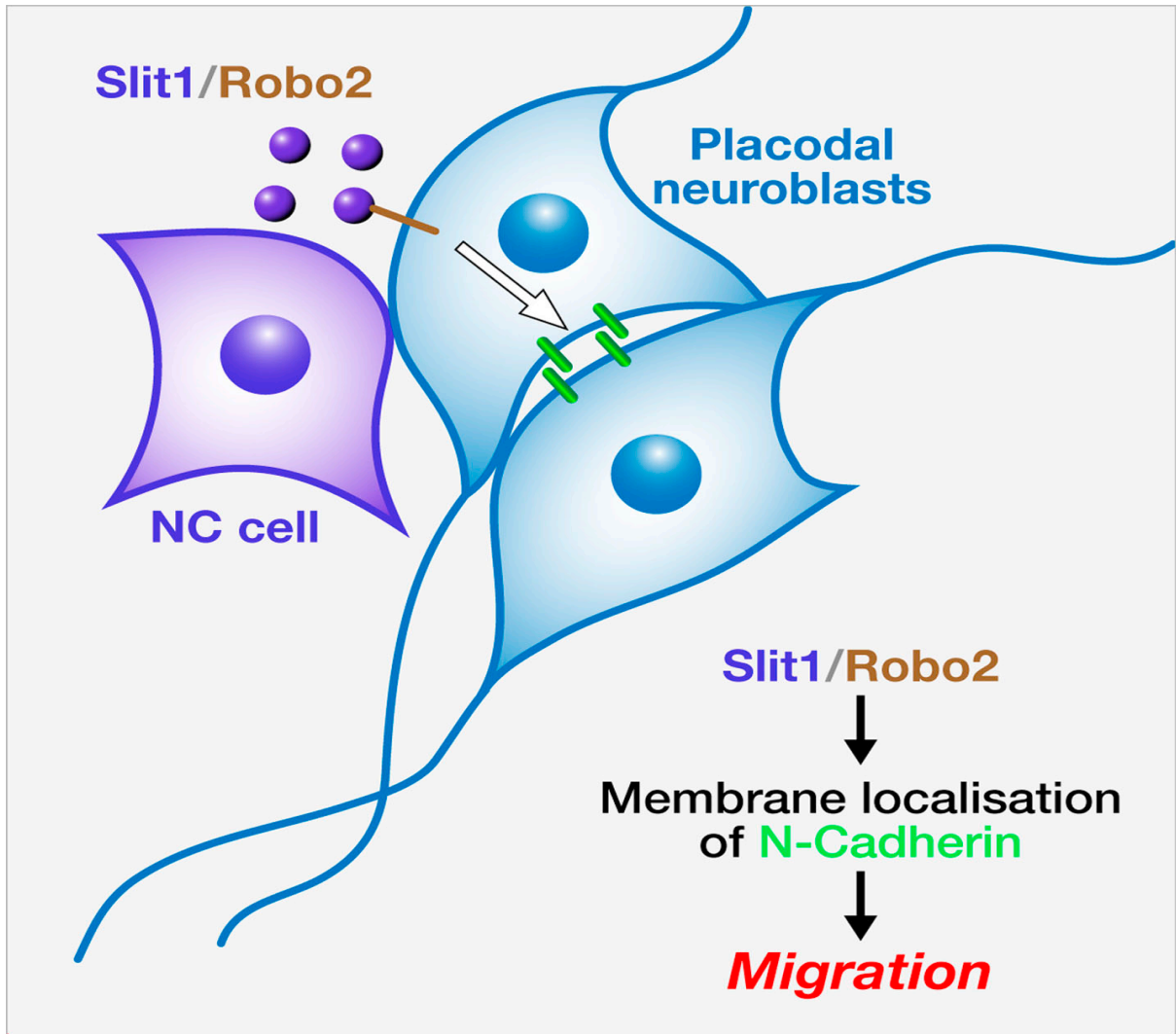


Fig. 1.4 Migration of placode cell-derived neurons requires the Slit/Robo signaling pathway. Slit1, a protein secreted by neural crest cells, activates Robo2 on placodal neurons/neuroblasts, which triggers localization of N-cadherin at membrane contact sites. This signaling pathway is essential for the migration of neurons. Adapted from (Breau and Schneider-Maunoury, 2015).

1.2.2 Cell Adhesion Molecules

Cell adhesion molecules (CAMs) are a family of proteins found on the plasma membrane of cells that aid in adhesion to other cells. There are at least five known classes of CAMs that include integrins, selectins, the immunoglobulin superfamily, cadherins, and mucins. CAMs are single pass transmembrane proteins with three conserved domains: an intracellular domain that interacts with the cytoskeleton, a transmembrane domain, and an extracellular domain that interacts with proteins on other cells (Harjunpää et al., 2019). Selectins and mucins modulate heterophilic interactions (i.e., between different types of proteins), while the immunoglobulin superfamily modulates homophilic interactions (i.e., between proteins of the same type). Cadherins are unique in that they can bind and interact with the same type of cadherin, making homophilic interactions, or bind to different types of cadherins, making heterophilic interactions. In contrast, integrins do not regulate intercellular adhesion, but instead bind to the components of the ECM. Two classes of the CAMs, selectins and cadherins, are calcium-dependent and contain binding sites for calcium ions that stabilize their adhesive interactions (Lodish, 2000). While each class of CAMs plays important roles in maintaining proper physiological conditions, cadherins are especially crucial during embryogenesis (including EMT and gangliogenesis) and will be discussed in greater detail below.

1.2.2.1 Adherens Junctions

Adherens junctions are one of the major structures necessary to form cell-cell adhesions (Fig. 1.5). These complexes are crucial during embryonic development, playing important roles in tissue morphogenesis, control of cell proliferation, polarity, shape, and motility of cells (Ivanov & Naydenov, 2013). When proper physical associations between epithelial cells are not maintained,

cell-cell contacts are lost, leading to disorganized tissue architecture (Meng & Takeichi, 2009). The core components of adherens junctions include classical cadherins, p120-catenin, β -catenin, and α -catenin. Interactions among these four proteins, the actin cytoskeleton, and microtubules control the formation, maintenance, and proper function of adherens junctions (Nagafuchi & Takeichi, 1988; McCrea & Gumbiner, 1991; Hirano et al., 1992; Hartsock & Nelson, 2008).

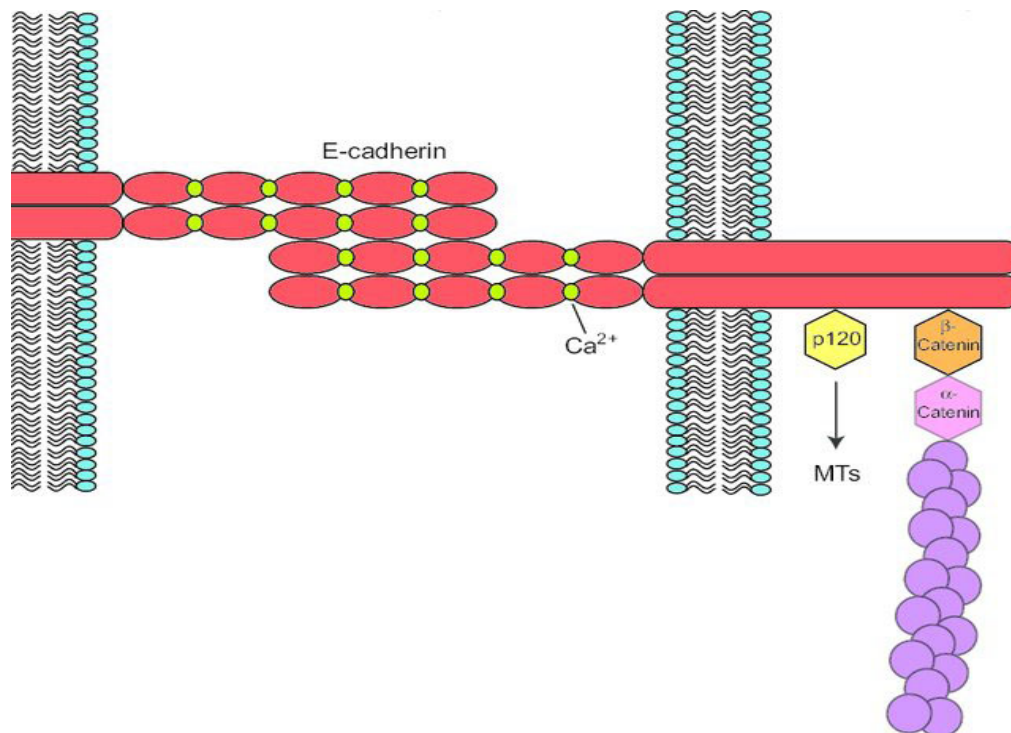


Fig. 1.5 The Cadherin-Catenin Complex.

The cadherin-catenin complex mediates interactions between cells and establishes apicobasal polarity. Cadherin molecules (here E-cadherin) present on adjacent cells first dimerize in “cis” and then in “trans” to form interactions that strengthen the adhesive properties of epithelial cells. The cytoplasmic domain of E-cadherin contains binding sites for p120- and β -catenin. While p-120-catenin directly binds to microtubules (MTs), β -catenin indirectly binds to actin, through interactions with α -catenin. These binding interactions allow adherens junctions to further strengthen cell-cell interactions. Adapted from (Lo et al. 2012).

1.2.2.2 Cadherins

Calcium-dependent adhesion proteins, abbreviated as cadherins, are a family of CAMs that play fundamental roles in tissue formation during development. Cadherins can be divided into four subclasses that exhibit distinct tissue distribution. The first subclass, the classical cadherins, are transmembrane glycoproteins containing an N-terminus extracellular domain and a C-terminus cytoplasmic domain (Shapiro & Weis, 2009). Classical cadherins contain five extracellular cadherin domains (EC1-EC5) with calcium binding sites between each domain. When calcium binds, cadherins homodimerize and mediate cell-cell adhesion (Ivanov et al., 2001). Two types of cadherin interactions exist: *cis* and *trans* (Fig. 1.6). *Cis* dimers form between cadherins on the same cell, while *trans* dimers form between cadherins on adjacent cells. While *trans* dimerization of cadherin proteins directly modulates cell adhesion, *cis* interactions are also important, as loss of the lateral lattice created by these interactions prevents adjacent cells from forming *trans* dimers (Harrison et al., 2011).

The specific cadherins present within adherens junctions vary by cell type and developmental context. For example, epithelial cells predominantly express epithelial or E-cadherin, whereas chick trigeminal neural crest and placode cells express cadherins such as Neural cadherin (N-cadherin), Cadherin-6B, and Cadherin-7 at different developmental timepoints. Prior to neural crest delamination, N-cadherin and Cadherin-6B are expressed in premigratory neural crest cells but are then later downregulated, while Cadherin-7 is upregulated in the migratory neural crest population (Taneyhill, 2008). Previous studies demonstrated that overexpression of either N-cadherin or Cadherin-7 affected neural crest cell migration, but not differentiation, indicating that cadherin expression regulates adhesiveness rather than specification (Nakagawa & Takeichi, 1998). Consistent with this, *in vitro* experiments show that N-cadherin-expressing S180

cells migrated significantly less and formed larger cell aggregations on fibronectin-coated plates than Cadherin-7-expressing cells, suggesting that N-cadherin mediates stable cell interactions while Cadherin-7 supports transient cell connections during motility (Dufour et al., 1999). In line with this, type I cadherins (E-cadherin and N-cadherin) exhibit much greater cell adhesiveness compared to type II cadherins (Cadherin-6B and Cadherin-7) (Chu et al., 2006a), further supporting the notion that the varying adhesive properties of adherens junctions involves the expression of different cadherins.

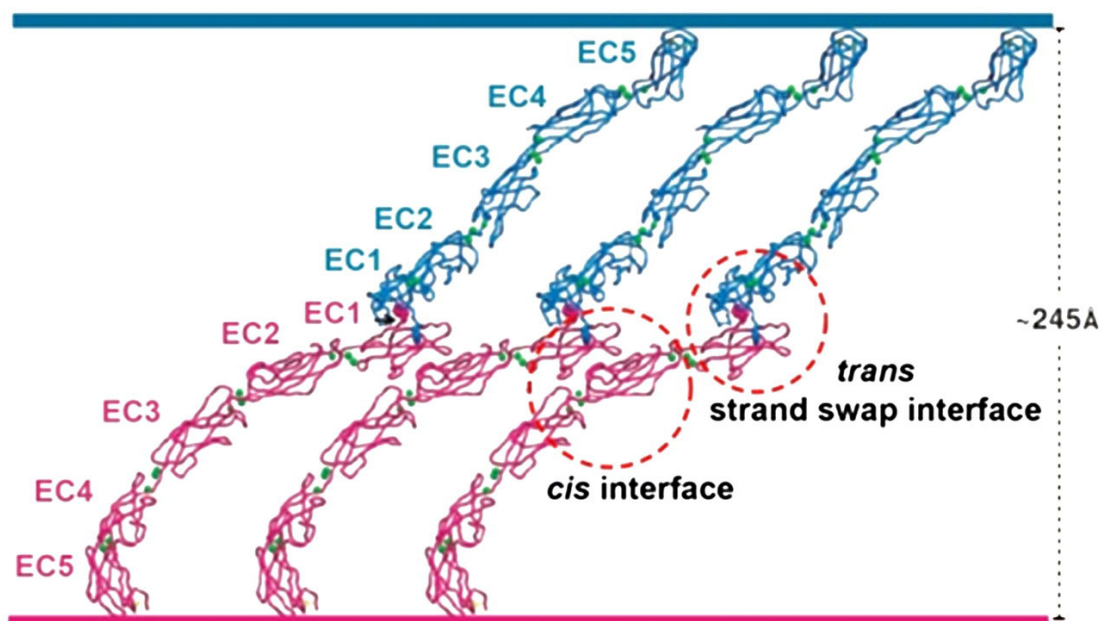


Fig. 1.6 *Cis* and *trans* dimer organization of classical cadherins.

Cadherin proteins contain 5 extracellular domains (EC1-EC5) which interact with domains on other cadherins to form connections. *Cis* interactions occur when the EC1 domain of one cadherin binds to a region between EC2 and EC3 on another cadherin in the same cell. This generates lateral interactions along the surface of one cell. *Trans* interactions occur when EC1 domains on adjacent cells bind to each other, anchored by Trp2. *Trans* interactions mediate cell-cell adhesion. Adapted from (Boggon et al., 2002).

Classical cadherins are most common during embryogenesis and include two subtypes: type I and type II. Type I classical cadherins contain a conserved HAV tripeptide motif in the most distal extracellular cadherin domain (EC1) and mediate strong adhesion (Halbleib & Nelson, 2006a). Members of this family include not only E- and N-cadherin but also Placental (P)-, Retinal (R)-, and Muscle (M)-cadherin (Paulson et al., 2014). In contrast, type II classical cadherins lack this motif and therefore mediate weaker adhesion. Members of this family include numerous cadherins named Cadherin-5 and beyond (Taneyhill, 2008). The other three subclasses of cadherins are desmosomal cadherins, protocadherins, and atypical cadherins. These subclasses function in mediating cell adhesion in tissues prone to mechanical stress (Shafraz et al., 2018), promoting cell motility in dynamic processes such as neuronal growth (Hayashi & Takeichi, 2015), and regulating cell polarity (Halbleib & Nelson, 2006a), respectively.

1.2.2.2a N-cadherin

N-cadherin, a type I classical cadherin, functions diversely during development, playing roles in cell convergence, maintenance of neuronal positioning, cell proliferation, and axonal pathfinding (Kerstetter et al., 2004). Initially discovered at the protein level in embryonic mice, (Hatta et al., 1985), N-cadherin was soon identified in neural retina tissue in the embryonic chick (Hatta & Takeichi, 1986). During early development in the chick, N-cadherin protein is detectable beginning at the primitive streak stage and persists through neurulation (Hatta & Takeichi, 1986). Subsequent work expanded on this by showing that both N-cadherin RNA and protein are present in all three germ layers, including mesodermal derivatives (Hatta et al., 1987a). In the chick, placode cells and their neuronal derivatives express N-cadherin prior to delaminating from the ectoderm (Shiau & Bronner-Fraser, 2009a). This early expression is driven in part by Sox2, a pro-

neural transcription factor, which is expressed in the neural tube and placode cells just prior to N-cadherin (Dady et al., 2012a; Matsumata et al., 2005). In line with this, previous studies have shown that N-cadherin possesses important roles during neurodevelopment including regulating neurite outgrowth (Riehl et al., 1996), axonal fasciculation (Masai et al., 2003), and proper synapse formation (Benson & Tanaka, 1998; Jontes et al., 2004). All of the listed examples highlight adhesion-dependent roles of N-cadherin during neurodevelopment; however, N-cadherin can also possess adhesion-independent functions such as controlling neural differentiation and acting as a modulator of various signaling pathways (Aubert et al., 2002; Punovuori et al., 2019; Shiau & Bronner-Fraser, 2009b).

1.2.2.2b Cadherin-7

Cadherin-7 is a type II classical cadherin that plays important roles in central nervous system development and neural crest migration. Although its function remains incompletely understood, its expression during embryogenesis is well described. Using the chick embryo as a model, Nakagawa and Takeichi (1995) discovered neural crest cells emerging from the neural tube to be Cadherin-7-positive. These cells maintained Cadherin-7 expression as they migrated to areas such as the dorsal root, the ventral root, and the spinal root (Nakagawa & Takeichi, 1995a). Studies in zebrafish further highlighted the importance of Cadherin-7, demonstrating stage-dependent expression in the notochord and more prolonged expression in the developing central nervous system (Liu et al., 2008). More recently, the expression of Cadherin-7 was established in the early forming cranial ganglia. In chick trigeminal ganglia, Cadherin-7 is solely expressed in the neural crest cell population of the ganglion, prior to their differentiation into neurons and glia, with no detectable expression in placode-derived neurons (C. Wu & Taneyhill, 2019). Perturbations to

Cadherin-7 resulted in morphological defects to the forming ganglion. These phenotypes resemble those reported by Shiau and Bronner-Fraser (2009), who did analogous experiments in the trigeminal ganglion with N-cadherin (Shiau & Bronner-Fraser, 2009). Together, this indicates Cadherin-7-expressing neural crest cells and N-cadherin-expressing placode-derived neurons play critical, complementary roles during trigeminal gangliogenesis, underscoring the importance of coordinated cadherin regulation in cranial sensory development.

1.2.2.3. Catenins

The catenins, a family of proteins including α -catenin, β -catenin, and p120-catenin, function together with cadherins to form the cadherin-catenin complex (Fig. 5). The intracellular domain of a cadherin is bound by both p120-catenin and β -catenin, the latter of which binds to α -catenin (Lo et al., 2012). Both p120-catenin and α -catenin interact with microtubules and actin filaments respectively, strengthening the adhesive properties of adherens junctions.

α -catenin can exist in either a monomeric or homodimeric state and acts as a molecular switch between the cadherin/ β -catenin complex and the actin cytoskeleton (Hartsock & Nelson, 2008). *In vitro* binding experiments have shown that monomeric α -catenin preferentially binds to the cadherin/ β -catenin complex, while homodimeric α -catenin preferentially binds F-actin (Drees et al., 2005; Yamada et al., 2005). More recent studies have shown that α -catenin interacts directly with vinculin, an actin-binding protein found in adherens junctions, to modulate the switch between binding the cadherin complex versus the actin cytoskeleton. Single molecule assays revealed that mechanical tension induces a conformational change in α -catenin to promote vinculin binding, promoting interactions with the actin cytoskeleton (C.-Y. Wu et al., 2014; Yao et al., 2014).

Originally identified as a segment polarity protein named armadillo in *Drosophila* (McCrea et al., 1991), β -catenin binds to the C-terminus of cadherins in a phosphorylation-dependent manner. It is a multifunctional protein that plays important roles in transcriptional activation as part of the Wnt pathway as well as in cadherin-mediated cell adhesion (Sineva & Pospelov, 2014). Canonical Wnt/ β -catenin signaling is essential for both neural crest and cranial placode development. In neural crest cells, this pathway sequentially and temporally controls neural crest induction, delamination, lineage specification, and differentiation of derivatives (Hari et al., 2012). Although the role of β -catenin in neural crest specification has been well established, its function in neural crest migration is less understood. Recent studies in *Xenopus* have suggested that canonical Wnt signaling contributes to neural crest migration, with perturbations to β -catenin disrupting migratory patterns (Maj et al., 2016). Wnt/ β -catenin signaling also plays an important role in embryonic stem cell-derived human neural crest cells, with studies indicating that Wnt levels determine axial identity of human neural crest cells in a dose-dependent manner (G. A. Gomez et al., 2019). In neurogenic placode cells, Wnt/ β -catenin signaling functions indirectly, working with the BMP and FGF pathways to contribute to the formation of the pre-placodal region (Kwon et al., 2010). Wnt signaling then regulates FGF signaling, which then in turn regulates the Notch pathway to drive the specification, differentiation, and delamination of neurons (Lassiter et al., 2014a).

p120-catenin contains a highly conserved peptide sequence that allows it to bind to the juxtamembrane domain of cadherins in adherens junctions. This interaction is thought to stabilize the cadherin at the plasma membrane to promote increased cell-cell adhesiveness during junction formation (Hartsock & Nelson, 2008). One way this stabilization is achieved is through the binding of p120-catenin to a motif in the cadherin cytoplasmic tail that signals for endocytosis. In chick

neural crest cells, the release of p120-catenin from Cadherin-6B triggers cadherin internalization to promote EMT (Padmanabhan & Taneyhill, 2015). This finding, along with others, illustrates a mechanism by which p120-catenin controls cadherin trafficking and adhesion during development (Bulgakova & Brown, 2016; Ireton et al., 2002; Nanes et al., 2012).

1.2.3 Neurotrophic factors and their receptors

Neurotrophic factors and their receptors play vital roles in neuronal survival, growth, and differentiation during the establishment of the nervous system (Fig. 1.7) (Huang & Reichardt, 2001). Neurotrophic signaling is not unique to the trigeminal ganglion nor sensory neurons, but functions in shaping both the central and peripheral nervous systems. Over 70 years ago, the first neurotrophic factor, nerve growth factor (NGF), was discovered when transplantation of a mouse sarcoma onto a developing chick embryo caused increased growth of sympathetic and sensory neurons. This was attributed to the presence of a previously unknown soluble factor secreted by the tumor (Levi-Montalcini & Hamburger, 1953). This observation led to the “Neurotrophic factor hypothesis,” which states that developing neurons compete for a limited amount of neurotrophic factors secreted from target tissues, ensuring that target tissues receive the proper innervation as the neurons that receive the factors survive, while the ones that do not die (Yuen et al., 1996). This hypothesis paved the way for the discovery of a family of proteins known as neurotrophins, which include NGF, brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT-3), and neurotrophin 4/5 (NT-4/5). These soluble factors are secreted from tissues that require neuronal innervation, and bind to their high affinity receptors expressed on the innervating neurons. These receptors, known as tropomyosin receptor kinases (Trks), are a subfamily of receptor tyrosine kinases (RTKs) that have specific ligand binding partners: NGF-TrkA, BDNF/NT-4/5-TrkB, and NT-3-TrkC (Blondy

et al., 2019) (Fig. 1.7). Additionally, all neurotrophins can bind to the neurotrophin receptor, p75, with similar affinities (Rodríguez-Tébar et al., 1991).

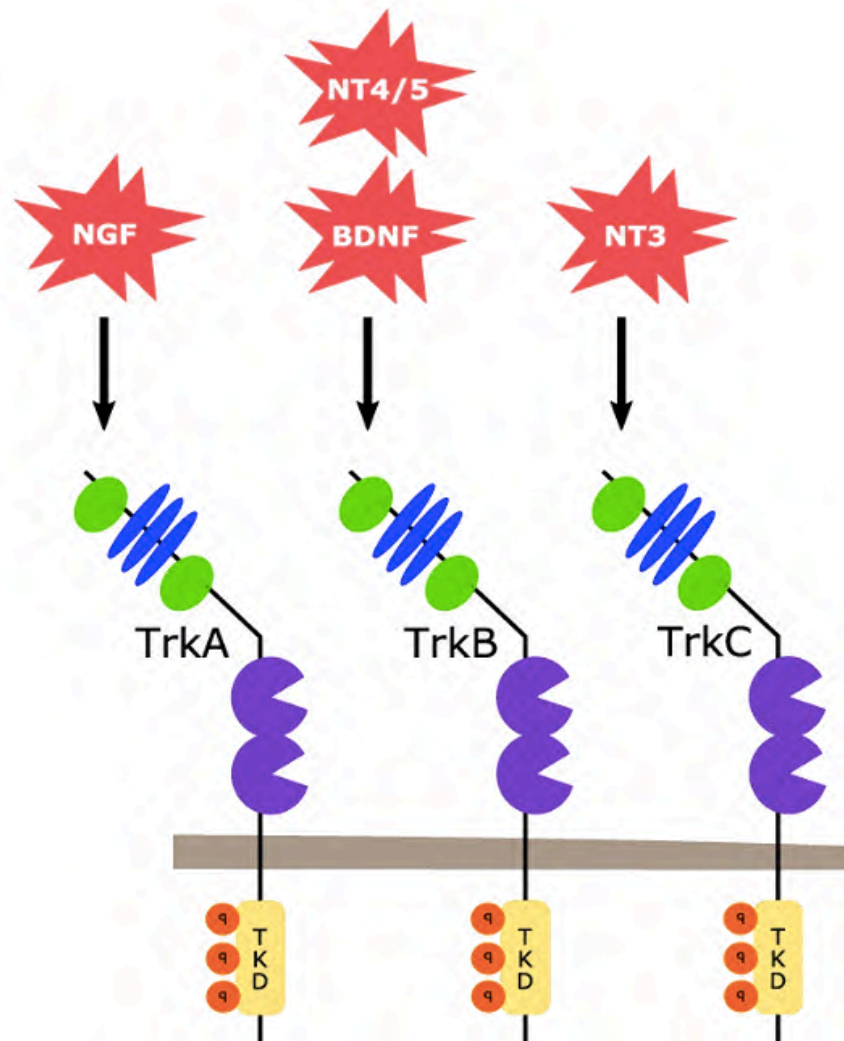


Fig. 1.7 Neurotrophic factors and their receptors.

Neurotrophic factors are family of proteins essential for neuronal development, survival, and function. These secreted factors bind to their cognate receptors, the tropomyosin receptor kinases (Trks) which are expressed on trigeminal neurons. Once bound, the Trks undergo autophosphorylation in their tyrosine kinase domain (TKD) to activate downstream signaling pathways including MAPK, PLC γ and PI3/Akt. Abbreviations: NGF, nerve growth factor; NT, neurotrophin; BDNF, brain-derived neurotrophic factor. Adapted from (Blondy et. al, 2019).

As RTKs, all neurotrophin-Trk interactions have similar mechanisms of action; however, the focus of this review will be NGF-TrkA signaling. The cytoplasmic tail of TrkA possesses a kinase domain containing tyrosine residues necessary for the activation of downstream signaling (Kaplan et al., 1991; Vetter et al., 1991). When NGF binds to TrkA, receptor dimerization occurs between two individual TrkA receptors. During this process, the kinase domains rotate into their active state, bringing the individual tyrosines into close proximity to each other. This rotation allows trans-autophosphorylation to occur, where each kinase domain gets phosphorylated by the adjacent kinase domain (Conroy & Coulson, 2022). This phosphorylation also generates docking sites for adaptor proteins, which are recruited to the intracellular domain of TrkA to activate downstream signaling pathways including MAPK, PI3K/Akt, and PLC γ (U. H. Kim et al., 1991; Miyasaka et al., 1992; Soltoff et al., 1992). Altogether, this promotes the survival and differentiation of neurons into different subclasses. Within the trigeminal ganglion, TrkA-expressing neurons become nociceptors, TrkB-expressing neurons become mechanoreceptors, and TrkC-expressing neurons become proprioceptors (Dykes et al., 2010).

In order for TrkA to reach the plasma membrane and act as a receptor for NGF, post-translational modifications, such as glycosylation, are essential and can influence its trafficking and stability at the cell membrane. Prior studies showed that TrkA undergoes extensive N-linked glycosylation within the endoplasmic reticulum, before getting additional sialic acid residues within the Golgi apparatus that contribute to its ability to act as a fully mature signaling receptor (Huang & Reichardt, 2003; Jullien et al., 2002; Watson et al., 1999). Additionally, experiments in sympathetic neurons revealed that the fully mature form of TrkA can be initially inserted into the plasma membrane of neuronal cell bodies before it gets endocytosed and trafficked to its growth cone, a process called transcytosis (Ascaño et al., 2009). The majority of the knowledge

surrounding TrkA glycosylation comes from studies using rat pheochromocytoma cells (PC12), since they endogenously express TrkA and undergo neuron-like differentiation in response to NGF (Greene & Tischler, 1976).

1.3 Cellular interactions mediating gangliogenesis

1.3.1 Heterophilic Cadherin Interactions

While cadherins are traditionally regarded as homophilic, there is accumulating evidence that non-like cadherins interact with each other, exhibiting heterophilic binding capabilities. These findings have been reported during normal development of the endoderm (Straub et al., 2011), in establishing synaptic potentials within the hippocampus (Basu et al., 2017), during *Xenopus* gastrulation (Prakasam et al., 2006), and in diseases such as cancer (Labernadie et al., 2017). Further *in vitro* studies have examined the binding affinities of type II cadherins and found that they engage in both homophilic and heterophilic interactions (Brasch et al., 2018). Most recently, studies in *Drosophila* have shown that the atypical cadherins Fat and Dachsous form heterodimers in adherens junctions during tissue morphogenesis. These heterophilic *trans* interactions regulate epithelial cell size dynamics, with loss of either leading to disruptions in complex formation and a reduction in tissue size (Kumar et al., 2019).

Whether such heterophilic cadherin complexes exist in the developing trigeminal ganglion remains unknown. Early work examining cadherin expression in chick neural crest cells suggested this possibility. Cadherin-7-expressing neural crest cells were found adjacent to other cells expressing N-cadherin and R-cadherin, indicating potential communication between distinct cadherin populations (Nakagawa & Takeichi, 1995a). Since this observation, little progress has been made in determining whether heterophilic interactions are present in the trigeminal ganglion.

While type I cadherins can form heterophilic interactions with other type I cadherins and type II cadherins can do the same, it is still not well understood whether type I and type II cadherins can establish heterophilic interactions with each other. These unanswered questions will be explored in this dissertation.

1.3.2 RTK-cadherin interactions

In contrast to the adhesion-dependent roles of cadherins that are largely mediated through their extracellular domains, cadherin intracellular domains function in relaying extracellular signals to elicit proper cellular responses. RTKs, which also sit at the cell surface, play important roles during development and, like cadherins, are often implicated in cancer. Cadherins and RTKs can influence each other's activity, which have been best studied with E-cadherin and EGF/EGFR (Jawhari et al., 1999; Ramírez Moreno & Bulgakova, 2021; Sullivan et al., 2022) and N-cadherin and FGF/FGFR signaling (Kon et al., 2019; Latko et al., 2019; Sanchez-Heras et al., 2006; E.-J. Williams et al., 2001) (Fig. 1.8 (Adam, 2015)). When a ligand binds to its RTK, the receptor autophosphorylates to activate downstream signaling pathways. The activation of RTKs has been shown to be capable of weakening cell adhesion. This is thought to occur by the RTK's ability to phosphorylate the catenins bound to the cytoplasmic domain of cadherins leading to their dissociation. The removal of p120-, β -, and α -catenin disrupts the link between a cadherin and the actin cytoskeleton, promoting the internalization of the cadherin, and subsequently weakening adhesion (Chiasson-MacKenzie & McClatchey, 2018a). Although this commonly occurs during tissue morphogenesis and collective cell migration in development (Leckband, 2023), it has also been observed in cancer (Russo et al., 2024; R. Xu et al., 2015). Stimulated RTKs expressed in the

leading edge of cells can promote endocytosis of cadherins to encourage migratory behaviors (Bertocchi et al., 2012; McCrea et al., 2015).

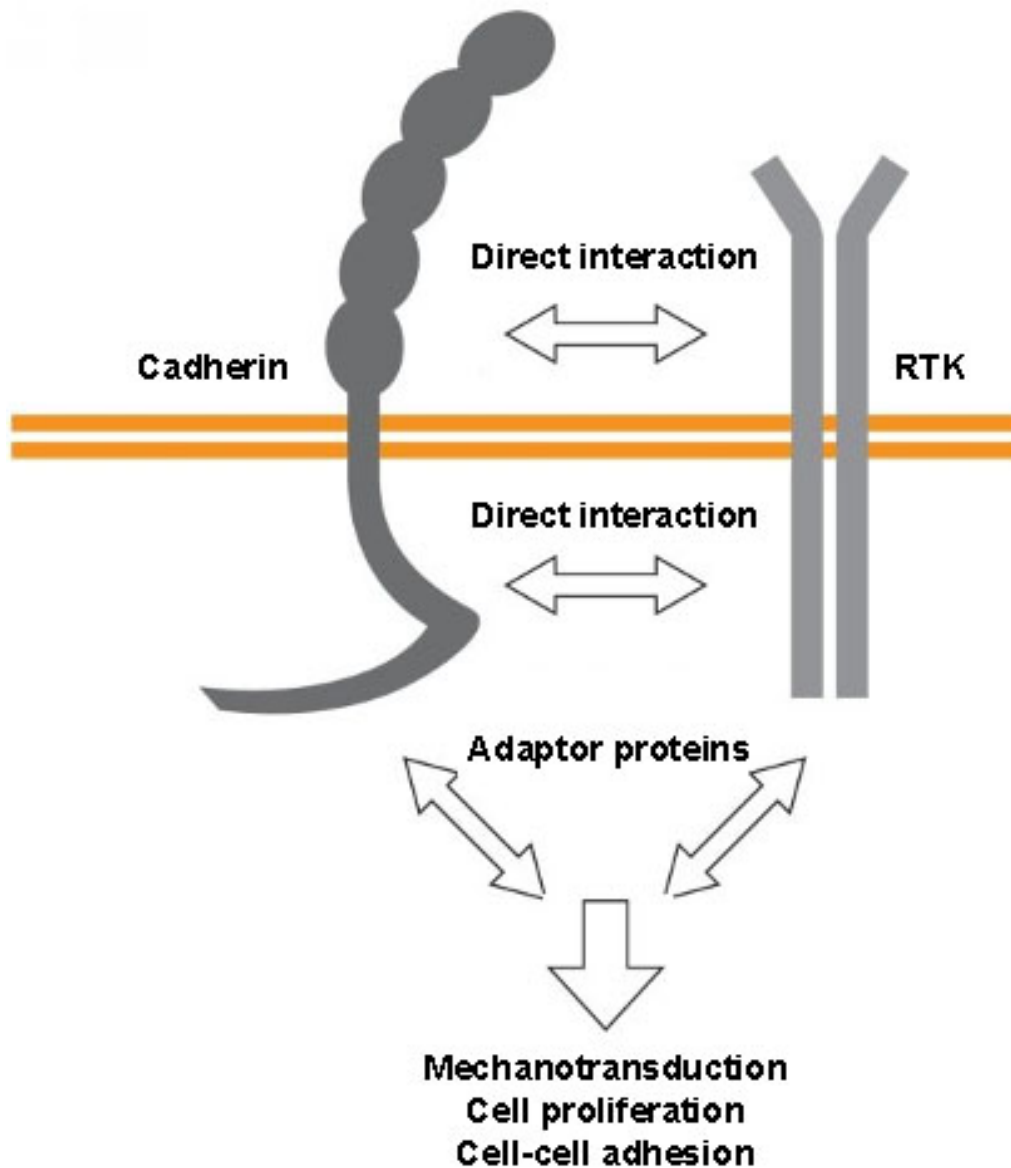


Fig. 1.8 Cadherin-RTK interactions at the membrane.

Cadherins and RTKs can regulate each other's activity in three main ways: direct interactions via their extracellular domains, direct interactions through their intracellular domains, or indirectly via adaptor proteins. Adapted from (Adam, 2015).

In contrast, cadherins can control signaling through RTKs using both their extracellular and intracellular domains. Cadherin extracellular domains have been shown to prevent signal transduction by blocking the binding site of the ligand on the RTK. Intracellularly, two methods of cadherin mediation of RTKs have been elucidated. Cadherin cytoplasmic domains can physically interact with the cytoplasmic domains of RTKs. Thus, if a cadherin is bound to an RTK, it can prevent the ligand/receptor complex from getting endocytosed, leading to a decrease in signaling activity. When RTKs get activated by their ligands, adaptor proteins are recruited to facilitate the activation of downstream pathways. In certain situations, adaptor proteins can preferentially bind to the cadherin/catenin complex over the RTK, preventing the activation of signal transduction through the RTK (Adam, 2015). Although these studies improve our understanding of the reciprocal interactions between cadherins and RTKs, further investigations about their roles in development are needed before we can fully understand how these systems get dysregulated in disease.

1.4 Conclusions and Future Implications

Growing evidence demonstrates cadherins as central regulators of trigeminal gangliogenesis, coordinating interactions between neural crest cells and placodal neurons as they assemble to form this structure and differentiate into specialized sensory neurons. Beyond their well-known roles in mediating homophilic adhesion, they can also selectively bind to different cadherins and receptors at the cell membrane. These additional roles suggest that cadherins play important roles beyond adhesion and serve as key molecules for intracellular communication and signaling during cranial ganglia formation.

While recent findings have established two cadherins, N-cadherin and Cadherin-7, to be expressed in the trigeminal ganglion in a lineage-restricted manner, it is not understood how these two proteins interact, nor the mechanism by which they do so during this developmental event. Even less understood are the interactions between cadherins and RTKs, and how these complexes mediate neuronal survival and subtype specification. Limited data exists on how cadherin-RTK interactions mediate proliferation and downstream signaling in other types of cells; however, whether these events occur in neurons during trigeminal gangliogenesis remains unknown. A more thorough investigation into how cadherin-mediated interactions (including heterophilic interactions with other cadherins and RTKs) impact gangliogenesis is essential to understanding the molecular events that underlie neural crest-placodal neuron communication. Such insights will not only clarify the multi-faceted roles cadherins play in cranial sensory development but may also reveal broader principles that adhesion molecules and signaling receptors utilize to coordinate the development of the nervous system.

Chapter 2: Neural crest cell-placodal neuron interactions are mediated by Cadherin-7 and N-cadherin during early chick trigeminal ganglion assembly

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F1000Research, 11, 741. <https://doi.org/10.12688/f1000research.122686.2>

2.1 Abstract

Arising at distinct positions in the head, the cranial ganglia are crucial for integrating various sensory inputs. The largest of these ganglia is the trigeminal ganglion, which relays pain, touch and temperature information through its three primary nerve branches to the central nervous system. The trigeminal ganglion and its nerves are composed of derivatives of two critical embryonic cell types, neural crest cells and placode cells, that migrate from different anatomical locations, coalesce together, and differentiate to form trigeminal sensory neurons and supporting glia. While the dual cellular origin of the trigeminal ganglion has been known for over 60 years, molecules expressed by neural crest cells and placode cells that regulate initial ganglion assembly remain obscure. Prior studies revealed the importance of cell surface cadherin proteins during early trigeminal gangliogenesis, with Cadherin-7 and neural cadherin (N-cadherin) expressed in neural crest cells and placode cells, respectively. Although cadherins typically interact in a homophilic (i.e., like) fashion, the presence of different cadherins expressed in neural crest cells and placode cells raises the question as to whether heterophilic cadherin interactions may also be occurring. Given this, the aim of the study was to understand whether Cadherin-7 and N-cadherin were interacting during initial trigeminal ganglion formation. To assess potential interactions between

Cadherin-7 and N-cadherin, we used biochemistry and innovative imaging assays conducted *in vitro* and *in vivo*, including in the forming chick trigeminal ganglion. Our data revealed a physical interaction between Cadherin-7 and N-cadherin. These studies identify a new molecular basis by which neural crest cells and placode cells can aggregate *in vivo* to build the trigeminal ganglion during embryogenesis.

2.2 Introduction

Cranial ganglia are sensory structures of the peripheral nervous system possessing the cell bodies of the cranial nerves. These ganglia and their associated nerves function in olfaction, taste, hearing, vision, and somatosensation (Gillig & Sanders, 2010; Sanders, 2010; Vermeiren et al., 2020). The trigeminal ganglion contains three sensory branches (ophthalmic, maxillary, and mandibular) that innervate different regions of the face to mediate sensations of pain, touch, and temperature (Davies, 1988; Gillig & Sanders, 2010; Higashiyama & Kuratani, 2014). During embryonic development, two distinct cell populations, neural crest cells and neurogenic placode cells, intermingle and aggregate to generate the trigeminal ganglion (Breau & Schneider-Maunoury, 2015; D'Amico-Martel & Noden, 1983; Hamburger, 1961b; Saint-Jeannet & Moody, 2014; Steventon et al., 2014). These interactions have been studied for over 60 years and reveal that each cell type contributes distinctly to trigeminal ganglion formation, with neural crest cells acting as a scaffold for the integration of placode cell-derived neurons, while placodal neurons aid in the condensation of neural crest cells (D'Amico-Martel & Noden, 1983; Hamburger, 1961b; Shiau et al., 2008b). Moreover, ablation of either of these cell populations leads to severe defects in trigeminal ganglion development, indicating a reciprocal relationship (D'Amico-Martel & Noden, 1983; Hamburger, 1961b; Lwigale, 2001).

Prior studies indicate that intercellular interactions during trigeminal ganglion formation are mediated, in part, by cadherin-based adhesion. Two cadherins, Cadherin-7 and neural cadherin (N-cadherin), are expressed in neural crest cells and placode cells, respectively, during chick trigeminal gangliogenesis. Expression of Cadherin-7, a type II classical cadherin, was discovered in migratory cranial neural crest cells in the chick embryo over 25 years ago (Nakagawa & Takeichi, 1995b). More recent studies of Cadherin-7 protein confirmed previous *in situ* hybridization findings and noted Cadherin-7 in chick migratory cranial neural crest cells contributing to the trigeminal ganglion (C. Wu & Taneyhill, 2019). Both depletion and overexpression of Cadherin-7 impact the distribution of embryonic neural crest cells and placodal neurons, and as such, the overall morphology of the ganglion. N-cadherin, a type I classical cadherin, is present throughout development and has been found in derivatives of the endoderm, mesoderm, and ectoderm (Hatta et al., 1987b). Notably, both ectodermal placode cells and their neuronal derivatives express N-cadherin (Shiau & Bronner-Fraser, 2009a) in the chick trigeminal ganglion. Knockdown of N-cadherin does not affect initial placode cell ingression and delamination from the ectoderm, but leads to increased placodal neuron dispersal during trigeminal gangliogenesis. Conversely, N-cadherin overexpression causes aberrant aggregation of placodal neurons (Shiau & Bronner-Fraser, 2009a). Modulation of N-cadherin levels appears to involve, in part, post-translational mechanisms linked to Slit1-Robo2 signaling in the developing chick trigeminal ganglion (Shiau & Bronner-Fraser, 2009a), but specific details underlying this process are not known.

While the ability of cadherins to make homophilic interactions is well understood, cadherins can also make heterophilic (i.e., non-like) connections with other cadherins, either in the same (homotypic) or different (heterotypic) cell types. Observations of heterophilic cadherin

interactions have been reported during normal development of the endoderm, (Straub et al., 2011) in establishing synaptic potentials within the hippocampus (Basu et al., 2017) and during *Xenopus* gastrulation (Prakasam et al., 2006) and are also noted in diseases such as cancer (Labernadie et al., 2017). In addition, the atypical cadherins Fat and Dachsous are capable of forming heterodimers between neighboring homotypic cells (Kumar et al., 2019). Collectively, these results support the notion that heterophilic interactions can occur between different types of cadherins during development. While previous studies noted the formation of aggregates from mixtures of N-cadherin- and Cadherin-7-expressing cells *in vitro* (Nakagawa & Takeichi, 1995b), the potential role of heterophilic cadherin interactions between neural crest cells and placode cell-derived neurons as they assemble the trigeminal ganglion has yet to be explored.

To address this question, we performed experiments to elucidate potential heterophilic interactions between Cadherin-7 and N-cadherin during the formation of the chick trigeminal ganglion. Our *in vivo* and *in vitro* biochemistry and imaging data indicate Cadherin-7 and N-cadherin physically interact during trigeminal ganglion assembly and that this involves heterophilic interactions between Cadherin-7, expressed in neural crest cells, and N-cadherin, found in placodal neurons. These findings further clarify the reciprocal relationship observed between coalescing neural crest cells and placodal neurons during trigeminal gangliogenesis, providing an additional molecular basis for this process.

2.3 Materials and Methods

Chick embryos

Fertilized chicken eggs (*Gallus gallus*) were obtained from the Department of Animal and Avian Sciences, University of Maryland, and Moyer's Chicks, Inc. (PA), and incubated at 37°C in

humidified incubators (EggCartons.com, Manchaug, MA, USA). Embryos were staged by the Hamburger-Hamilton (HH) staging method (Hamburger & Hamilton, 1992) or by counting the number of somite pairs (somite stage, ss).

Ethical approval

No ethical approval was required for this study for the chick embryos. The NIH Office for Protection from Research Risks has interpreted “live vertebrate animal” to apply to avians (e.g., chick embryos) only after hatching. Since our work does not utilize hatched chicks, no Institutional Animal Care and Use protocol for this work is necessary.

Green fluorescent protein (GFP) reconstitution across synaptic partners (GRASP) cadherin construct preparation

Four different GRASP constructs were synthesized by GenScript (RRID:SCR_002891) to allow for incorporation of split GFP moieties (subunits 1-10 or subunit 11) into the extracellular domain of Cadherin-7 and N-cadherin, with the design based on similar plasmids generated in (Yamagata & Sanes, 2012) and available in Addgene (m-sGFP1-10::NLG1 (Addgene plasmid #44967; RRID:Addgene_44967) and m-sGFP11::NXN were gifts from Joshua Sanes (Addgene plasmid #44968; RRID: Addgene_44968)). Briefly, each plasmid from Addgene was modified to remove the respective insert (either NLG1 or NXN), and, in its place, we inserted the Cadherin-7 or N-cadherin cDNA sequence corresponding to the mature peptide. Sequence accuracy of constructs was confirmed by GenScript and expression of each cadherin was validated through immunocytochemistry.

Cell culture and transfection assays

Chinese hamster ovary (CHO) cells (ATCC Cat# CCL-61, RRID:CVCL_0214; American Type Culture Collection) were cultured in Ham’s F12 media (10-080, Corning/Cellgro) supplemented

with 10% fetal bovine serum (Genesee Scientific Cat#25-514H). Transient transfection assays were carried out using the Lipofectamine 2000 reagent (Thermo Fisher Scientific, Inc., Cat#11668019). Cells were grown to 90% confluency, and transfections were performed according to the manufacturer's instructions and according to the protocols outlined in (Schiffmacher et al., 2014, 2016). The chick N-cadherin expressing (pCIG-N-cadherin) and empty (pCIG) vectors were gifts from Dr. Marianne Bronner (California Institute of Technology).

Immunoprecipitations

Embryonic trigeminal ganglia were used for immunoprecipitations, with tissue harvested as described previously by (Schiffmacher et al., 2016; Shah et al., 2017; C. Wu & Taneyhill, 2019). Briefly, forming trigeminal ganglia were dissected, pooled, pelleted, flash-frozen in liquid nitrogen, and stored at -80°C. Cultured cells were scraped into 1X Phosphate-buffered Saline (1X PBS), pelleted, flash-frozen in liquid nitrogen, and stored at -80°C. Pellets were thawed on ice and lysed in lysis buffer (50mM Tris pH 8.0, 150mM NaCl, 1% IGEPAL CA-630) supplemented with cOmplete protease inhibitor cocktail tablets (Roche, Cat#04693124001) and 1 mM PMSF (Sigma Aldrich Cat#10837091001) for 30 minutes at 4°C with periodic mixing. Soluble fractions were collected following centrifugation at maximum speed for 15 minutes at 4°C (Microfuge 20R Centrifuge, Beckman Coulter, Inc., Cat#B31612), and protein concentration was quantified (BioPhotometer, Eppendorf, Cat#6131 26936) by Bradford assay (Thermo Fisher Scientific, Inc., Cat#1863028). Immunoprecipitations were carried out using protein A/G magnetic beads (Thermo Fisher Scientific, Inc., Cat#88802) according to the manufacturer's instructions (Thermo Fisher Scientific, Inc.). Equivalent amounts of protein lysates (~120 µg) were incubated with 10 µg rabbit polyclonal N-cadherin antibody (Abcam Cat#ab12221, RRID:AB_298943) or normal rabbit IgG control (R&D Systems Cat#AB-105-C, RRID:AB_354266) overnight at 4°C with constant

rotation. The following day, 0.25 mg washed protein A/G magnetic beads were incubated with the lysate/antibody mixture for one hour at room temperature with mixing. Following incubation, the samples were washed, equivalent volumes of SDS sample buffer were added, mixtures were boiled at 100°C for 10 minutes, magnetic beads were collected, and samples were loaded for immunoblotting as described below. Input amounts represent 5% (trigeminal ganglia) and 10% (cell culture) of the initial lysate amount used in the immunoprecipitation. Assays were conducted twice.

Immunoblotting

Immunoblotting after immunoprecipitation was performed according to the protocol by (Schiffmacher et al., 2016; Shah et al., 2017; C. Wu & Taneyhill, 2019). Samples were processed via SDS-PAGE (10% Mini-Protean TGX gel, BioRad #456-1034) in 1X Running Buffer (25 mM Tris (Thermo Fisher Scientific, Inc., Cat#BP-152-1), 192 mM glycine (Thermo Fisher Scientific, Inc., Cat#AC120070010), 0.1% sodium dodecyl sulfate (VWR, Cat#4095-02)) and then transferred to 0.45 µm BioTrace nitrocellulose membrane (Pall, Cat#66485) via wet transfer (Biorad, Mini-PROTEAN Tetra Vertical Cell for Mini Precast gels, Cat#1658004) in 1X Transfer Buffer (Running Buffer + 10% Methanol (Thermo Fisher Scientific, Inc., Cat#A452-4)) according to the manufacturer's guidelines. For immunoblotting, membranes were blocked in blocking buffer (1X PBS + 0.1% Tween-20 (Sigma Aldrich, Cat#P1379-500ML)) (PTW) + 5% non-fat milk (Carnation Instant Nonfat Dry Milk). Next, primary antibodies against mouse monoclonal Cadherin-7 (1:150, DSHB, Cat#ccd7-1, RRID:AB_528111) or rabbit polyclonal N-cadherin (1:1000, Abcam Cat#ab12221) were diluted as indicated in blocking buffer and incubated overnight with shaking at 4°C. Unbound primary antibodies were washed off with PTW (three times, 10 minutes each), followed by incubation at room temperature for 45 minutes with the

following secondary antibodies diluted in blocking buffer (1:10,000): goat anti-mouse polyclonal IgG (H&L) antibody peroxidase conjugated (Rockland Cat# 610-1302, RRID: AB_219656) or goat anti-rabbit polyclonal IgG (H&L) secondary antibody peroxidase conjugated (Rockland Cat# 611-1302, RRID:AB_219720). After washing three times, 10 minutes each, in PTW, proteins were detected using enhanced chemiluminescent substrates mixed in a 1:1 ratio (SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific, Inc., Cat#34580) or SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, Inc., Cat#34095)). Immunoblot images for figures were gamma-modified and processed using Adobe Photoshop (RRID:SCR_014199) CC 2019 (20.0.6 release, Adobe Systems, San Jose, CA, USA).

Immunostaining

Embryos collected at various stages, or cultured cells in two-well chamber slides (LAB-TEK, Cat#154461), were used for immunostaining. For the former, detection of various proteins was performed on 14 μ m transverse sections following 4% paraformaldehyde (PFA) fixation overnight, gelatin embedding, and cryostat sectioning, according to the protocol described previously by (Shah et al., 2017; C. Wu & Taneyhill, 2019; C.-Y. Wu et al., 2014). For the latter, cells were fixed in 4% PFA for 15 minutes, followed by immunocytochemistry. Tissue or cells were permeabilized by washing two times, 10 minutes each, in 1X PBS + 0.1% Triton X-100 (Sigma Aldrich, Cat#TX1568-1) (PBSTX), followed by a one-hour blocking step of PBSTX + 10% sheep serum (Sigma Aldrich, Cat#S2263-100ML). All primary and secondary antibodies were diluted in 1X PBSTX + 5% sheep serum. The following antibodies and dilutions were used for immunostaining: mouse monoclonal anti-Cadherin-7 (1:50-1:70, DSHB Cat#ccd7-1); rat monoclonal anti-N-cadherin (1:50, DSHB Cat#MNCD2, RRID:AB_528119); mouse monoclonal antihuman natural killer-1 (HNK-1) (1:100, DSHB Cat#3H5, RRID:AB_2314644); and mouse

monoclonal anti-Tubulin beta-3 chain (Tubb3) (1:500, Abcam Cat# ab78078, RRID:AB_2256751). The following secondary antibodies were used at 1:200-1:500 dilutions: goat anti-mouse polyclonal IgG (H + L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 594 (Thermo Fisher Scientific Cat# A-11005, RRID:AB_2534073) and goat anti-mouse polyclonal IgG (H + L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 647 (Thermo Fisher Scientific Cat# A-21235, RRID:AB_2535804) (for Cadherin-7); goat anti-rat polyclonal IgG (H + L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 594 (Thermo Fisher Scientific Cat# A-11007, RRID:AB_10561522) (for N-cadherin); goat anti-mouse polyclonal IgM (Heavy Chain) Secondary Antibody (Thermo Fisher Scientific Cat# A-21238, RRID:AB_2535807) (for HNK-1); and goat anti-mouse polyclonal IgG2a Human ads-AF555 (SouthernBiotech Cat# 1080-32, RRID:AB_2794491) (for Tubb3). Sections were stained with 40,6-diamidino-2-phenylindole (DAPI) to mark cell nuclei using DAPI-containing mounting media (DAPI Fluoromount-G, Southern Biotech, Cat#0100-20).

In ovo electroporation

For sequential electroporation of both premigratory neural crest cells and trigeminal placode cells, unilateral chick neural tube electroporation to target neural crest cells contributing to the trigeminal ganglion was first performed, as described previously by (C. Wu & Taneyhill, 2019; C.-Y. Wu et al., 2014). Briefly, GRASP constructs were introduced unilaterally into premigratory midbrain neural crest cells in developing 3 to 5 somite stage (3-5ss) chick embryos at a concentration of 2.0-2.5 µg/µl, using fine glass needles to fill the chick neural tube. Platinum electrodes were placed on either side of the embryo, and two 25 V, 25 ms electric pulses were applied across the embryo. Once embryos reached HH10-11 (10-13ss), a unilateral ectodermal electroporation was carried out (on the same side of the embryo that was electroporated previously) to target trigeminal placode

cells.²⁶ Electrodes were placed vertically on top of and below the embryo and three, 9 V pulses were delivered over 50 ms at 200 ms intervals. After electroporation, eggs were re-sealed with tape and parafilm and re-incubated for the desired time period (approximately 36 hours to reach HH15-16) prior to harvesting for fixation and transverse sectioning, which was carried out according to the protocol by.¹⁴ Unilateral co-electroporation of N-cadherin split GFP constructs into trigeminal placode cells was carried out as described (Shah et al., 2017).

Confocal imaging

For all experiments, images of at least five serial transverse sections through a minimum of three embryos, or eight cell culture images/replicates, were acquired with the LSM Zeiss 800 confocal microscope with Airyscan detection (Carl Zeiss Microscopy, Thornwood, NY, USA) at 20X magnification. Laser power, gain, and offset were kept consistent for the different channels during all experiments where possible. ZEN Digital Imaging for Light Microscopy (RRID: SCR_013672), version 2.3 software (Carl Zeiss Microscopy) and Adobe Photoshop CC 2019 (20.0.6 release) were used for image processing. For the *in vivo* GRASP experiments, images were false-colored in Adobe Photoshop to allow for better visualization and comparison of signal. Equivalent functions for image processing can be performed on Fiji (RRID: SCR_002285), which is freely available.

Quantification

Images from both *in vitro* and *in vivo* experiments were analyzed using the Squassh (Rizk et al., 2014) plugin from the MOSAICSuite in FIJI (Schindelin et al., 2012). Segmentation parameters were adjusted per experiment, to minimize interfering background signal. For the *in vitro* assays, the amount of GFP positive and DAPI positive cells were counted and divided by each other to determine transfection efficiency. For N-cadherin *in vivo* control experiments, GFP positive cells

and N-cadherin positive cells were counted and divided by each other to determine electroporation efficiency. For the *in vivo* heterophilic cadherin experiments, the number of GFP regions were counted per section, and averaged to find the average amount of GFP-positive regions. After batch analysis, the quality of image-segmentation was confirmed visually in order to ensure accurate segmentation.

2.4 Results

2.4.1 Heterophilic cadherin interactions exist in the forming chick trigeminal ganglion

Cranial neural crest cells and trigeminal placodal neurons express distinct cadherins, Cadherin-7 and N-cadherin, respectively, during early trigeminal gangliogenesis (Shiau & Bronner-Fraser, 2009a; C. Wu & Taneyhill, 2019). Given these findings, we sought to determine whether these specific cadherins facilitated trigeminal ganglion assembly through heterophilic interactions. To address this, we performed co-immunoprecipitation assays on lysates prepared from dissected forming trigeminal ganglia of HH15-16 chick embryos (Fig. 2.1) (McCabe et al., 2007). Analysis of immunoprecipitated proteins by SDS-PAGE followed by immunoblotting for N-cadherin revealed detection of N-cadherin within the forming trigeminal ganglia (Fig. 2.1A, lane 2) and in N-cadherin immunoprecipitates with this antibody (Fig. 2.1A, lane 4), but not with the rabbit IgG serum (Fig. 2.1A, lane 3). These data indicate that the N-cadherin antibody can effectively immunoprecipitate N-cadherin from trigeminal ganglia tissue, providing us with a key experimental tool to identify other proteins that physically interact with N-cadherin *in vivo*. To this end, we next performed immunoblotting using a validated Cadherin-7 antibody (Nakagawa & Takeichi, 1995b; C. Wu & Taneyhill, 2019) (Fig. 1B). Our data again reveal a band corresponding to Cadherin-7 observed in the trigeminal ganglia lysate input sample (Fig. 2.1B, lane 1,

arrowhead), along with immunoreactive lower molecular weight bands (Fig. 2.1B, asterisk, *) containing portion(s) of the Cadherin-7 extracellular domain, as observed in our prior work (C. Wu & Taneyhill, 2019). Strikingly, we also observed Cadherin-7 after N-cadherin pull-down (Fig. 2.1B, lane 4, arrowhead), but not with the control IgG serum (Fig. 2.1B, lane 3). These findings reveal N-cadherin and Cadherin-7 physically interact *in vivo*. As N-cadherin is noted in trigeminal placodal neurons and cranial mesenchyme (Shiau & Bronner-Fraser, 2009a) but only neural crest cells express Cadherin-7 (C.-Y. Wu et al., 2014), our data suggest heterophilic interactions between Cadherin-7 in neural crest cells and N-cadherin in placodal neurons and/or the mesenchyme.

2.4.2 Cadherin-7 and N-cadherin form heterophilic interactions *in vitro*

Given the results of our pull-down experiments, we hypothesized that physical interactions between Cadherin-7 in neural crest cells and N-cadherin in placodal neurons mediate, in part, the successful aggregation of these cell types during trigeminal gangliogenesis. To address this, we adapted and modified a GRASP assay to evaluate interactions specifically between these two cadherins, both *in vitro* and *in vivo*. GRASP relies upon functional complementation (i.e., GFP fluorescence) between two non-fluorescing or split GFP fragments (GFP1-10, GFP11). Reconstitution of GFP can only occur when the split GFP molecules are in close proximity to each other, as observed in other systems that defined interactions between extracellular domains of two membrane proteins (Feinberg et al., 2008; Feng et al., 2016; Roy et al., 2014; Yamagata & Sanes, 2012). We designed Cadherin-7 and N-cadherin GRASP vectors (Fig. 2.2) with GFP subunits fused in frame to the respective cadherin extracellular domain (Cadherin-7 GFP1-10, Cadherin-7 GFP11, N-cadherin GFP1-10, N-cadherin GFP11; GenScript). Constructs were based on GRASP

plasmids developed by the Sanes lab (Addgene), which generate intact GFP fluorescence due to neuroligin-neurexin interactions, with no GFP noted with single constructs (Yamagata & Sanes, 2012).

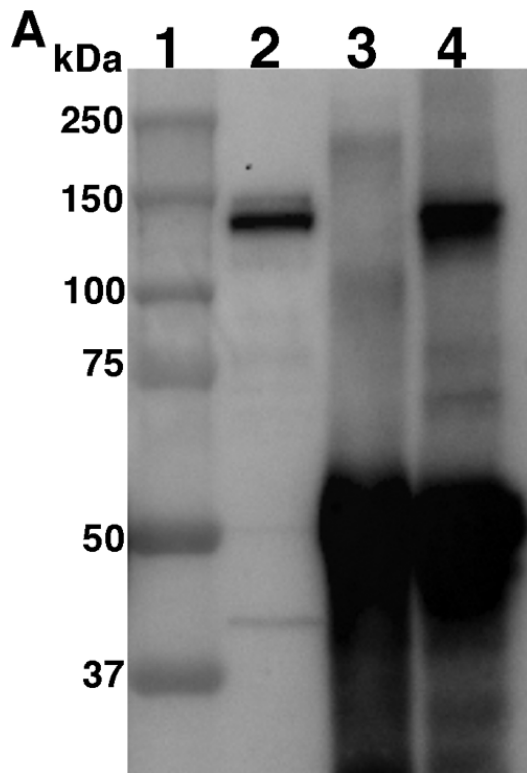
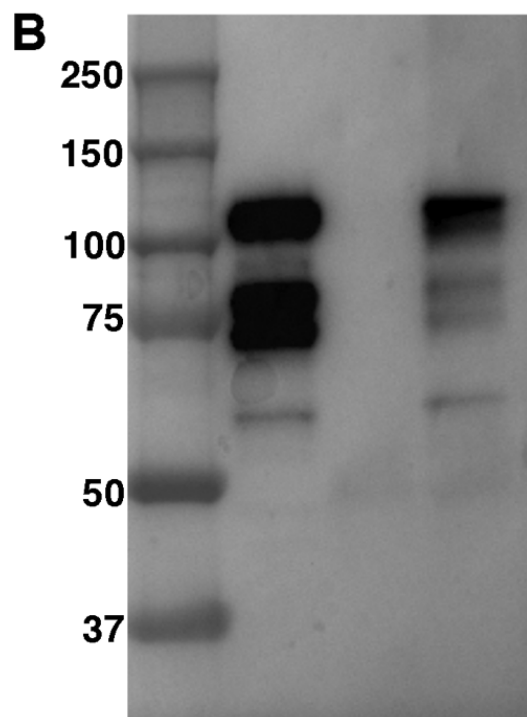


Fig. 2.1 Cadherin-7 and N-cadherin physically interact *in vivo*.

Lysate from HH15-16 trigeminal ganglion tissue was incubated with either an antibody against N-cadherin or with whole rabbit IgG serum as a control. Immunoprecipitated proteins were captured with protein A/G beads, separated by SDS-PAGE, followed by immunoblotting for N-cadherin (A) and Cadherin-7 (B). Lanes 1-4 are as follows: 1) Protein ladder; 2) Input, trigeminal ganglia lysate; 3) trigeminal ganglia lysate following IP with rabbit IgG; and 4) trigeminal ganglia lysate following IP with N-cadherin antibody. Arrowheads point to N-cadherin (A) or Cadherin-7 (B), respectively, while asterisks identify Cadherin-7 immunoreactive products as observed previously (C. Wu & Taneyhill, 2019). N=2 experiments. N-cadherin, neural cadherin; IP, immunoprecipitation. Contributions from C. Wu.



We first showed that all constructs expressed their respective cadherins by transfecting CHO cells, which lack endogenous cadherins (Hong et al., 2010), with each GRASP construct, followed by immunostaining for each cadherin (Fig. 2.3A', B', C', D', arrows). Importantly, no GFP fluorescence was noted under any condition, as expected. We next evaluated the specificity of the split GFP moieties to generate GFP by co-transfecting CHO cells with the same split GFP constructs, but fused to a different cadherin (i.e., Cadherin-7 GFP1-10 and N-cadherin GFP1-10). In these control experiments, expression of each cadherin was observed once again (Fig. 2.4A'', B'', arrows), but no GFP was reconstituted, reinforcing the specificity of the assay.

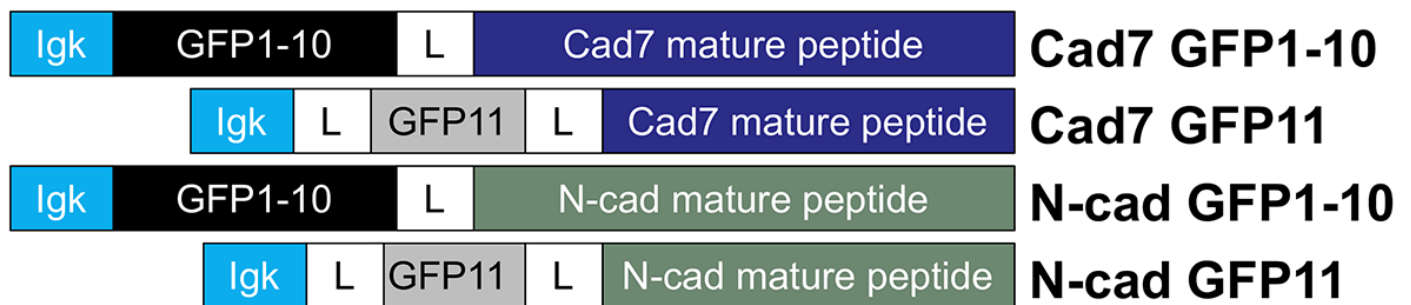


Fig. 2.2 Cadherin-expressing GRASP constructs.

Cartoon diagram showing GFP-cadherin fusion proteins that were constructed by joining the kappa light chain to distinct GFP subunits (1–10, or 11), followed by a linker region and then the mature cadherin peptide. GFP, green fluorescent protein; GRASP, GFP reconstitution across synaptic partners; N-cad, neural cadherin; Cad7, Cadherin-7.

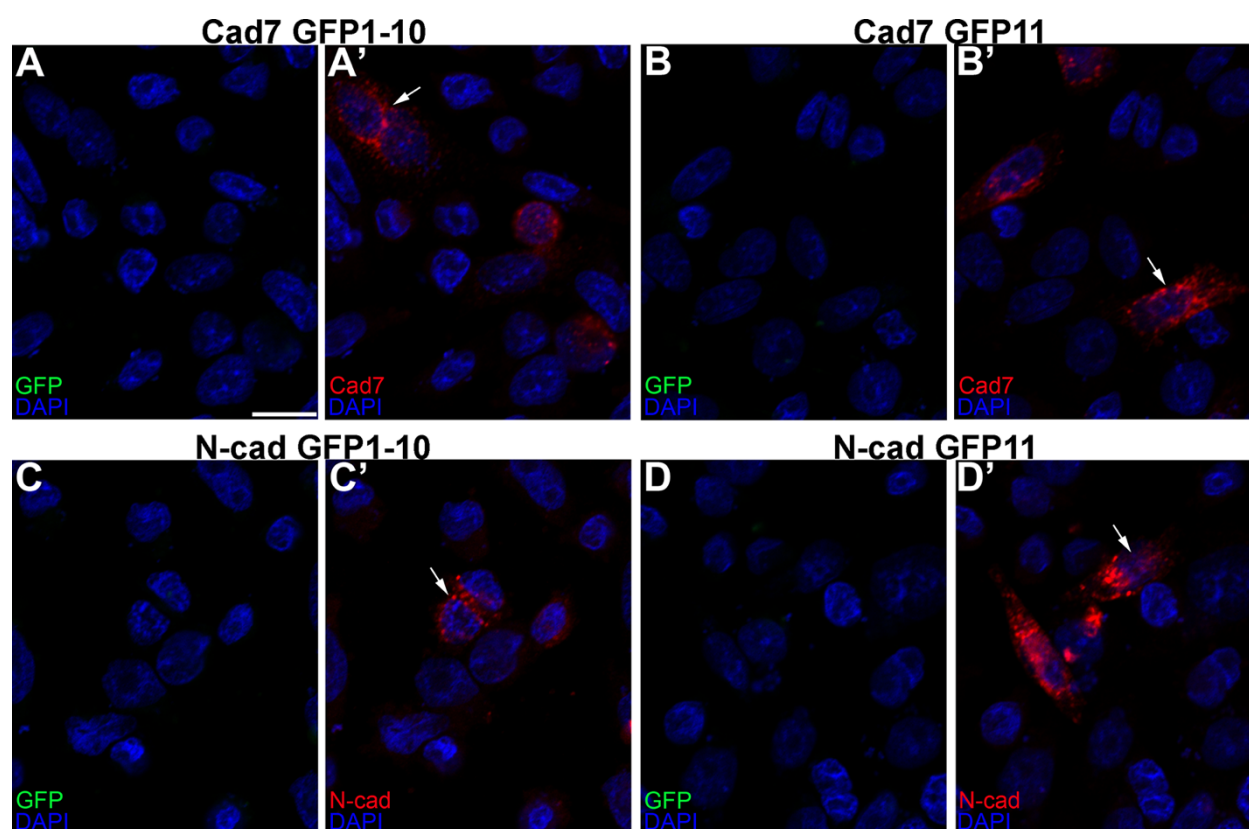


Fig. 2.3 Individual cadherin GRASP constructs express their respective cadherins but not GFP.

Single transfections of Cad7 GFP1-10 (A-A'), Cad7 GFP11 (B-B'), N-cad GFP1-10 (C-C'), and N-cad GFP11 (D-D') were conducted in CHO cells, followed by immunocytochemistry for Cad7 (A', B', red) or N-cad (C', D', red). GFP fluorescence was also examined in the appropriate microscope channel (488) but not observed. Arrows point to cadherin expression in transfected cells. DAPI (blue), cell nuclei. N=3 replicates for all treatments. Scale bar in (A) is 50 μ m and applies to all images. GFP, green fluorescent protein; GRASP, GFP reconstitution across synaptic partners; CHO, Chinese hamster ovary; N-cad, neural cadherin; Cad7, Cadherin-7. Contributions from C. Wu.

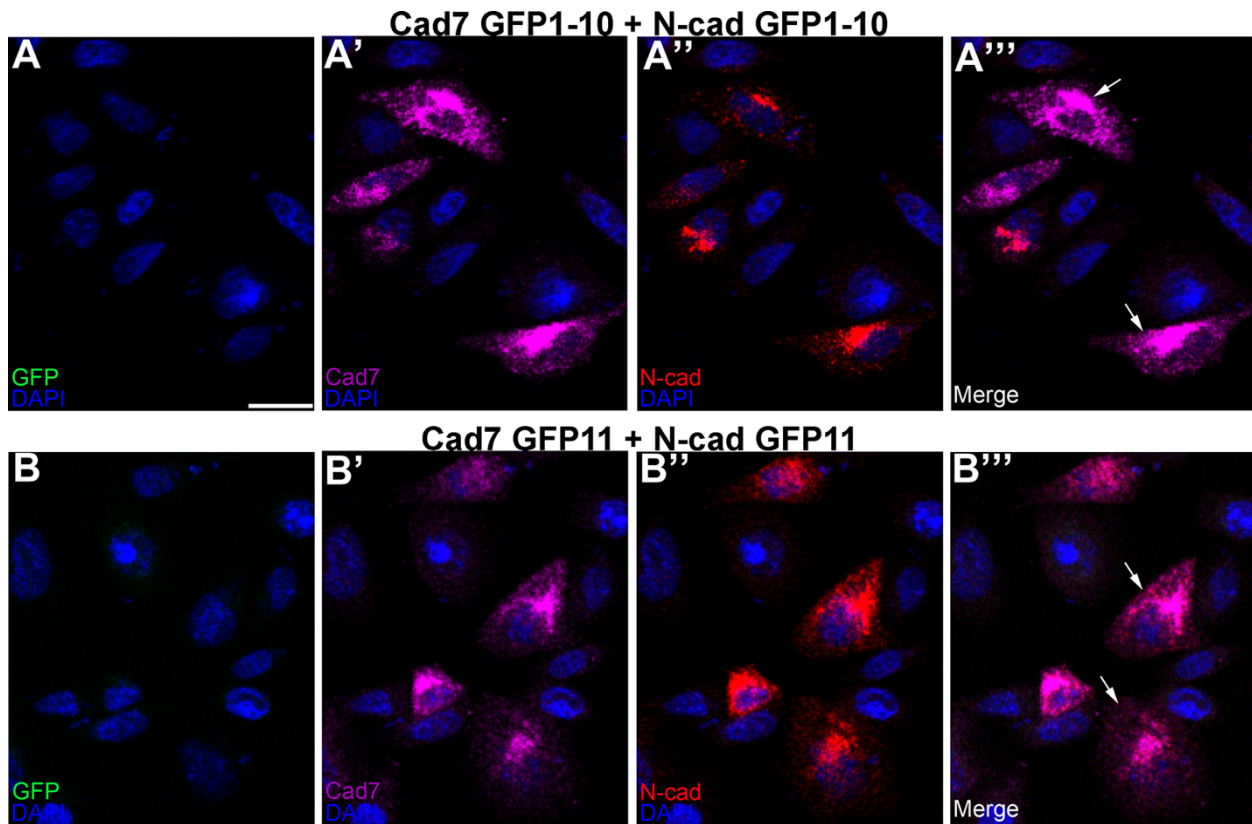


Fig. 2.4 Different cadherin-expressing GRASP constructs possessing the same GFP domains do not reconstitute GFP.

Co-transfection of CHO cells with Cad7 GFP1-10 + N-cad GFP1-10 (A-A'''), or Cad7 GFP11 + N-cad GFP11 (B-B'''), was performed, followed by immunocytochemistry for Cad7 (A', A'', B', B'', purple) and N-cad (A'', A''', B'', B'', red). GFP fluorescence was also examined in the appropriate microscope channel (488) but not observed. Arrows point to cadherin expression in co-transfected cells. DAPI (blue), cell nuclei. Scale bar in (A) is 50 μ m and applies to all images. N=8 replicates for both treatments. GFP, green fluorescent protein; GRASP, GFP reconstitution across synaptic partners; CHO, Chinese hamster ovary; N-cad, neural cadherin; Cad7, Cadherin-7. Contributions from C. Wu.

Next, we addressed whether *cis* interactions between complementary split GFP constructs could generate an intact GFP molecule *in vitro*. To this end, we co-transfected CHO cells with complementary split GFP constructs expressing the same cadherin (Fig. 2.5) and examined GFP fluorescence and cadherin expression by immunostaining. GFP fluorescence was detected with both Cadherin-7- (Fig. 2.5A, A'', arrows) or N-cadherin- (Fig. 2.5B, B'', arrows) expressing split GFP constructs, along with expression of each respective cadherin (Fig. 2.5A', B'), demonstrating effective GFP reconstitution via homophilic cadherin interactions. Further, these transfected cells exhibited a round morphology with few protrusions emanating from the cell surface, similar to that seen with control transfections.

Next, we calculated the percentage of GFP-positive cells in all of our transfection assays and observed a greater number of GFP-positive cells after transfection of homophilic split cadherin GFP constructs compared to that observed after transfection of heterophilic split cadherin GFP constructs (Table 2.1). While there is no statistically significant difference in the percentage of GFP-positive cells in N-cad GFP1-10 + N-cad GFP11- versus Cad7 GFP1-10 + Cad711- transfected cells ($p=0.19$), we did find a statistically significant difference in the percentage of GFP-positive cells after transfection of heterophilic cadherin split GFP constructs. Transfection of Cad7 GFP1-10 + N-cad GFP11 gave rise to a 1.5-fold increase in the percentage of GFP-positive cells compared to N-cad GFP1-10 + Cad7 GFP11 ($p=0.013$).

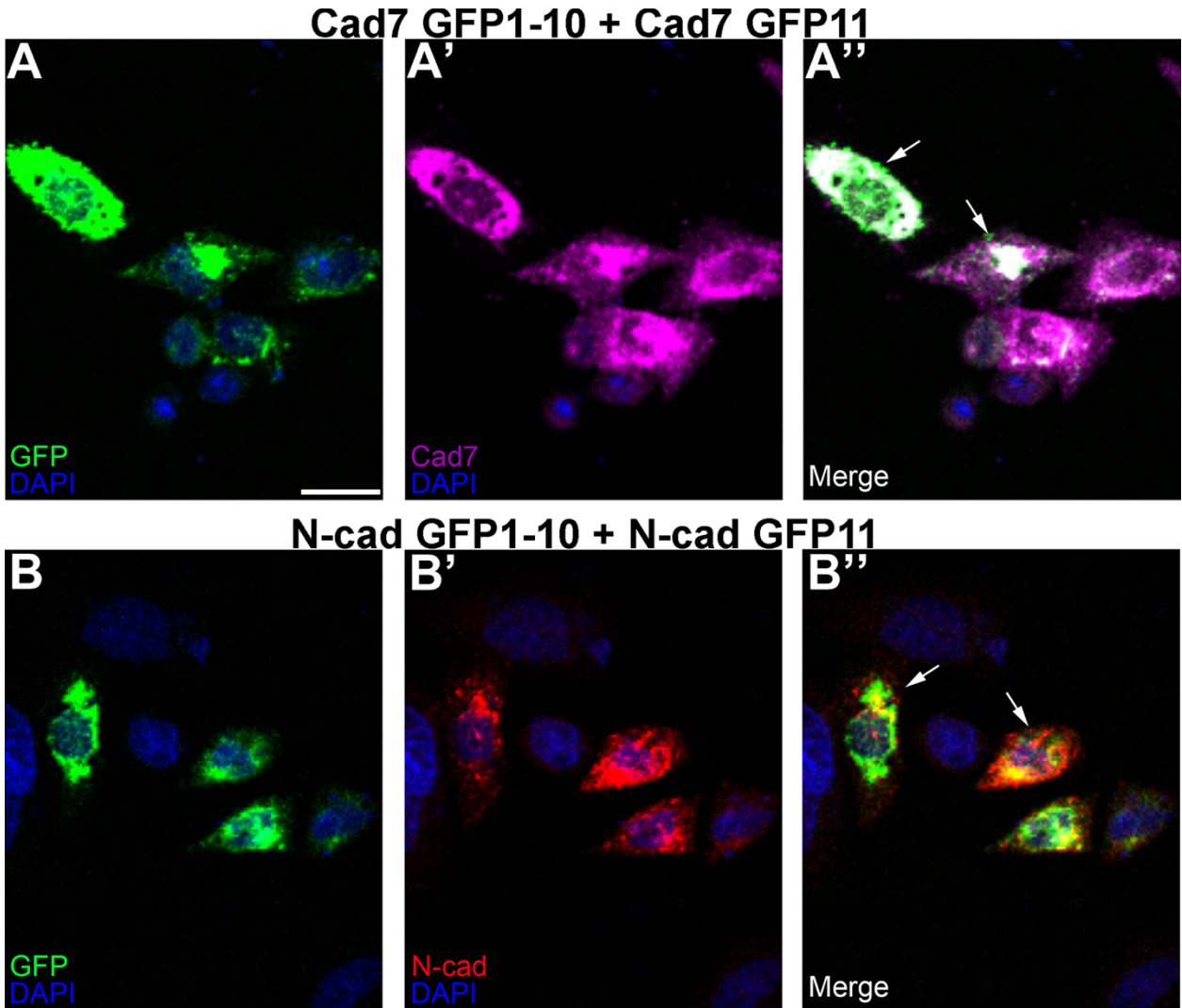


Fig. 2.5 Identical cadherin-expressing GRASP constructs possessing complementary GFP domains reconstitute GFP in *cis*.

CHO cells were co-transfected with complementary split GFP constructs expressing the same cadherin (Cad7 GFP1-10 and Cad7 GFP11 (A-A''); N-cad GFP1-10 and N-cad GFP11, (B-B'')), followed by immunocytochemistry for Cad7 (A', A'', purple) or N-cad (B', B'', red). GFP fluorescence was also examined in the appropriate microscope channel (488, A, A'', B, B'', green). Arrows point to GFP fluorescence in transfected cells, indicative of physical interactions between each split GFP-expressing cadherin. DAPI (blue), cell nuclei. Scale bar in (A) is 50 μm and applies to all images. N=8 replicates for both treatments. GFP, green. Contributions from C. Wu.

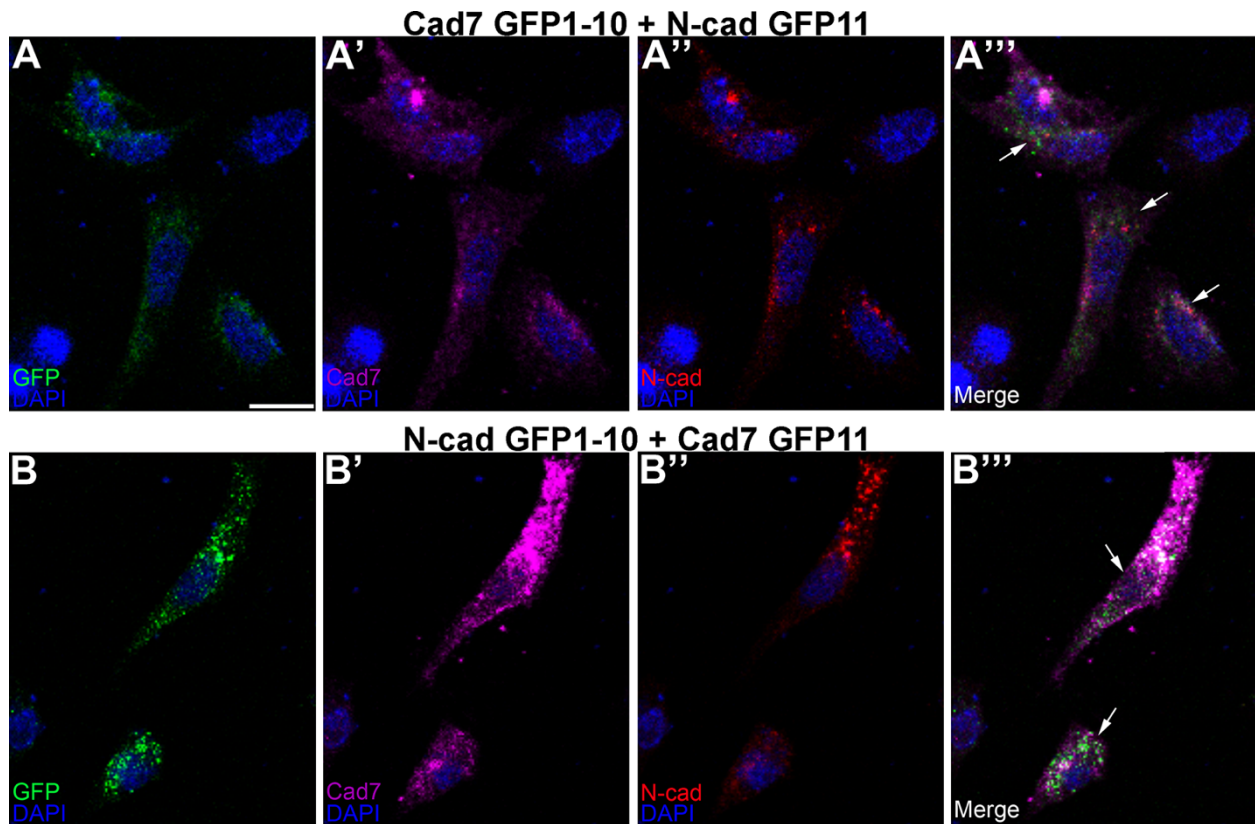


Fig. 2.6 Different cadherin-expressing GRASP constructs possessing complementary GFP domains reconstitute GFP *in cis*.

Cad7 GFP1-10 and N-cad GFP11 (A-A'''), or N-cad GFP1-10 and Cad7 GFP11 (B-B'''), were co-transfected into CHO cells, followed by immunocytochemistry for Cad7 (A', A'', B', B'', purple) and N-cad (A'', A''', B'', B''', red). GFP fluorescence was also examined in the appropriate microscope channel (488, A, A''', B, B''', green). Arrows point to GFP fluorescence in transfected cells, indicative of physical interactions between each split GFP-expressing cadherin. DAPI (blue), cell nuclei. Scale bar in (A) is 50 μ m and applies to all images. N=8 replicates for both treatments. GFP, green fluorescent protein; GRASP, GFP reconstitution across synaptic partners; CHO, Chinese hamster ovary; N-cad, neural cadherin; Cad7, Cadherin-7. Contributions from C. Wu.

	Cad7 GFP1-10+ Cad7 GFP11	N-cad GFP1-10+ N-cad GFP 11	Cad7 GFP1-10 + N-cad GFP11	Cad7 GFP11 + N- cad GFP1-10
Percent transfected	10.51±0.22% ^a	13.22±0.91% ^a	8.90±0.64% ^a	5.76±0.88% ^b

Table 2.1 Transfection efficiency of Cadherin split GFP plasmids.

2.4.3 Physical interactions between neural crest cells and placodal neurons in the trigeminal ganglion are mediated, in part, by Cadherin-7 and N-cadherin

To corroborate our findings and examine cadherin intercellular interactions during trigeminal ganglion assembly *in vivo*, we turned to a sequential electroporation assay in which a Cadherin-7 split GFP construct was first electroporated into premigratory neural crest cells, followed by a second electroporation of a complementary N-cadherin split GFP construct to target trigeminal placode cells in the surface ectoderm (Fig. 2.7 and 2.8). Transverse sections taken from electroporated embryos were processed for immunohistochemistry to identify neural crest cells and placodal neurons within the forming trigeminal ganglion. Remarkably, we observed GFP-positive regions and/or puncta (Fig. 2.7E-G'; Fig. 2.8E-G'; arrows) between neural crest cells (labeled by HNK-1; Fig. 2.7B, D, E, G, G'; Fig. 2.8B, D, E, G, G') and placodal neurons (labeled by Tubb3; Fig. 2.7C, D, F, G, G'; Fig. 2.8C, D, F, G, G') in the presence of the appropriate split GFP constructs. These data indicate Cadherin-7 and N-cadherin are in close proximity to interact *in trans* and permit the reconstitution of GFP *in vivo*, even in different cell types.

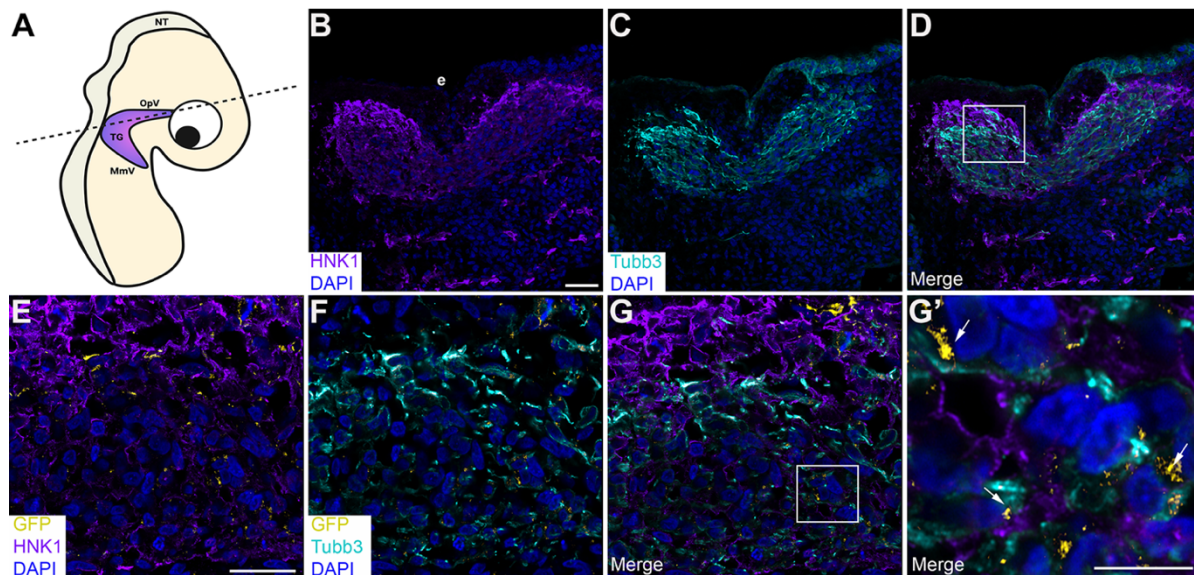


Fig. 2.7 Cadherin-7 GFP1-10 and N-cadherin GFP11 form heterophilic interactions in *trans* in the trigeminal ganglion.

(A) Diagram of a chick embryo showing the trigeminal ganglion, with the dotted line indicating the axial level at which images were captured (BioRender.com). Premigratory neural crest cells were electroporated with Cad7 GFP1-10, followed by placode cell electroporation with N-cad GFP11. Section immunohistochemistry for HNK1 (purple, marks neural crest cells; B, D, E, G, G') and Tubb3 (cyan, marks neurons which are placode cell-derived at this stage; C, D, F, G, G') was then performed. GFP signal (yellow, arrows, E-G') was captured in the appropriate channel (488). (B-D) Representative sections of the morphology of the trigeminal ganglion at this stage of development. (E-G) Higher magnification images of the boxed region in (D). (G') Higher magnification image of the boxed region in (G), with GFP identified by arrows. DAPI (blue), cell nuclei. Scale bar in (B) is 100 μ m and applies to (C-D); scale bar in (E) is 100 μ m and applies to (F-G); and scale bar in (G') is 50 μ m. N=6 embryos. NT, neural tube; MmV, maxillomandibular lobe; OpV, ophthalmic lobe; TG, trigeminal ganglion; e, ectoderm; N-cad, neural cadherin; Cad7, Cadherin-7; GFP, green fluorescent protein; HNK-1, human natural killer-1; Tubb3, Tubulin beta-3 chain.

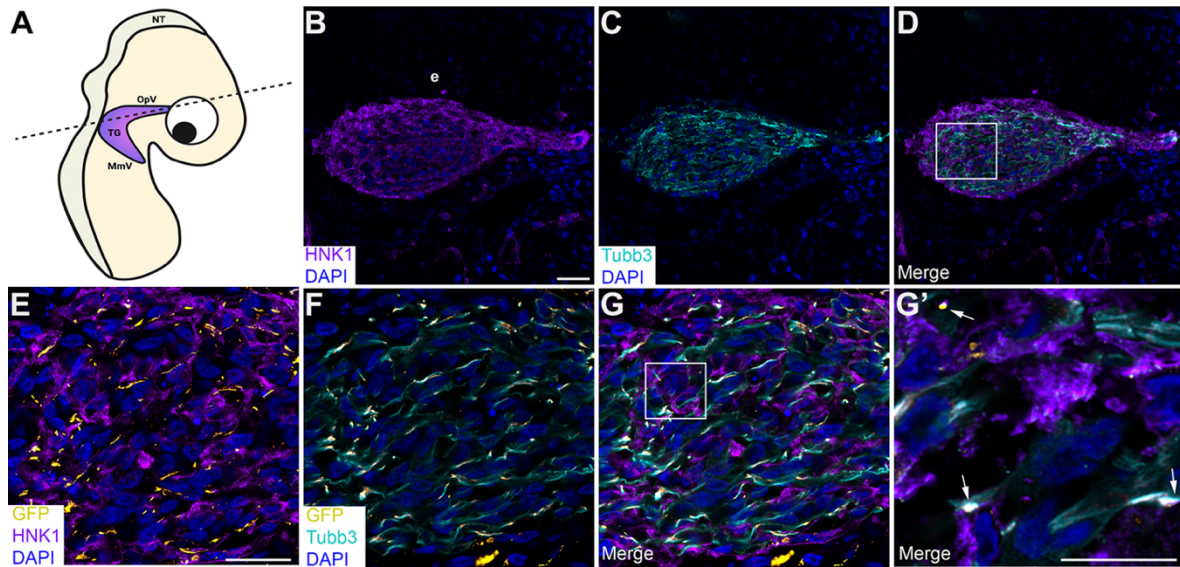


Fig. 2.8 Cadherin-7 GFP11 and N-cadherin GFP1-10 form heterophilic interactions in trans in the trigeminal ganglion.

(A) Diagram of a chick embryo showing the trigeminal ganglion, with the dotted line indicating the axial level at which images were captured (BioRender.com). Premigratory neural crest cells were electroporated with Cad7 GFP11, followed by placode cell electroporation with N-cad GFP1-10. Section immunohistochemistry for HNK1 (purple, marks neural crest cells; B, D, E, G, G') and Tubb3 (cyan, marks neurons which are placode cell-derived at this stage; C, D, F, G, G') was then performed. GFP signal (yellow, arrows, E-G') was captured in the appropriate channel (488). (B-D) Representative sections that show the morphology of the trigeminal ganglion at this stage of development. (E-G) Higher magnification images of the boxed region in (D). (G') Higher magnification image of the boxed region in (G), with GFP identified by arrows. DAPI (blue), cell nuclei. N=13 embryos. Scale bar in (B) is 100 μ m and applies to (C-D); scale bar in (E) is 100 μ m and applies to (F-G); and scale bar in (G') is 50 μ m. NT, neural tube; MmV, maxillomandibular lobe; OpV, ophthalmic lobe; TG, trigeminal ganglion; e, ectoderm; N-cad, neural cadherin; Cad7, Cadherin-7; GFP, green fluorescent protein; HNK-1, human natural killer-1; Tubb3, Tubulin beta-3 chain.

	N-cad GFP1-10; N-cad GFP11	Cad7 GFP1-10+ N-cad GFP11	Cad7 GFP11 + N-cad GFP1-10
GFP-positivity	53±0.29%	16 regions±2.07 ^a	10 regions±1.42 ^b

Table 2.2 Electroporation efficiency of Cadherin split GFP plasmids.

Next, we quantified the number of GFP-positive regions/puncta in serial sections through the forming trigeminal ganglion in each sequential electroporation experimental condition. Remarkably, this analysis yielded similar results to what we observed *in vitro*, namely a 1.6-fold increase in the number of GFP-positive regions/puncta after sequential electroporation of Cad7 GFP1-10 + N-cad GFP11 compared to that seen in Cad7 GFP11 + N-cad GFP1-10-electroporated embryos ($p=0.014$, Table 2.2). In contrast, co-electroporation of N-cad GFP1-10 + N-cad GFP11 into trigeminal placode cells, followed by incubation of embryos to trigeminal ganglion-forming stages, yielded robust GFP fluorescence throughout the plasma membrane of placodal precursors (Fig. 2.9A-C) and neurons (Fig. 2.9D', arrows). Quantification of the number of GFP-positive cells in these experiments revealed that 53% of the N-cadherin-positive trigeminal placode cells and neurons were also GFP-positive when split GFP construct electroporations were performed *in cis* (Table 2.2). These data are consistent with those seen *in vitro* after transfection of the same cadherin split GFP construct combination. Together with our biochemistry data and results in cultured cells, our findings support the assertion that heterophilic interactions between Cadherin-7 in neural crest cells and N-cadherin in placodal neurons occur during trigeminal gangliogenesis.

Moreover, the preceding data are in keeping with the *in vivo* distribution of N-cadherin-expressing placodal neurons and Cadherin-7-expressing neural crest cells in the forming ganglion at this developmental stage (Fig. 2.10). Although it is evident that neural crest cells and placodal

neurons are in close proximity and can interact (Fig. 2.10B', arrows), the distribution of these cells also reveals that homophilic cadherin interactions are likely to occur within each cell type proper (i.e., neural crest cell-neural crest cell, placodal neuron-placodal neuron) as the ganglion begins to assemble. Collectively, our *in vivo* results support a role for neural crest cell-placode cell interactions during early trigeminal ganglion development.

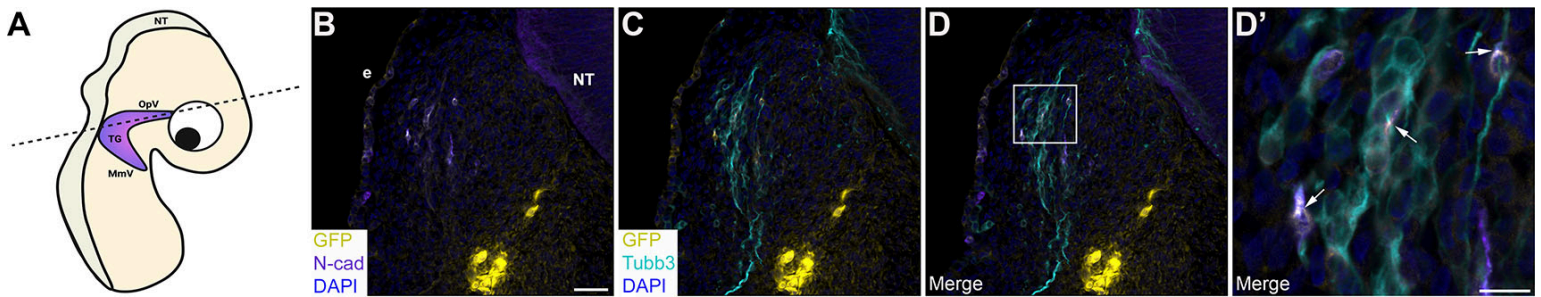


Fig. 2.9 N-cadherin GRASP constructs expressing complementary GFP domains reconstitute GFP in *cis* in the trigeminal ganglion.

(A) Cartoon diagram of a chick embryo showing the forming trigeminal ganglion, with the dotted line indicating the axial level at which images were captured. Diagram created with BioRender.com. To target trigeminal placode cells, ectodermal electroporations were conducted in the chick embryo with both N-cad GFP1-10 and N-cad GFP11 constructs. Section immunohistochemistry for N-cad (purple; B, D, D') and Tubb3 (cyan, marks neurons placode cell-derived at this stage; C-D') was then performed. GFP signal (yellow, arrows, B-D') was captured in the appropriate channel (488). (B-D) Representative sections that show the morphology of the trigeminal ganglion at this stage in development. (D') Higher magnification image of the boxed region in (D), with GFP and N-cad double-positive cells identified by arrows. Scale bar in (B) is 100 μm and applies to (C-D), and scale bar in (D') is 50 μm . N=3 embryos. NT, neural tube; MmV, maxillomandibular lobe; OpV, ophthalmic lobe; TG, trigeminal ganglion; e, ectoderm; N-cad, neural cadherin; GFP, green fluorescent protein; Tubb3, Tubulin beta-3 chain.

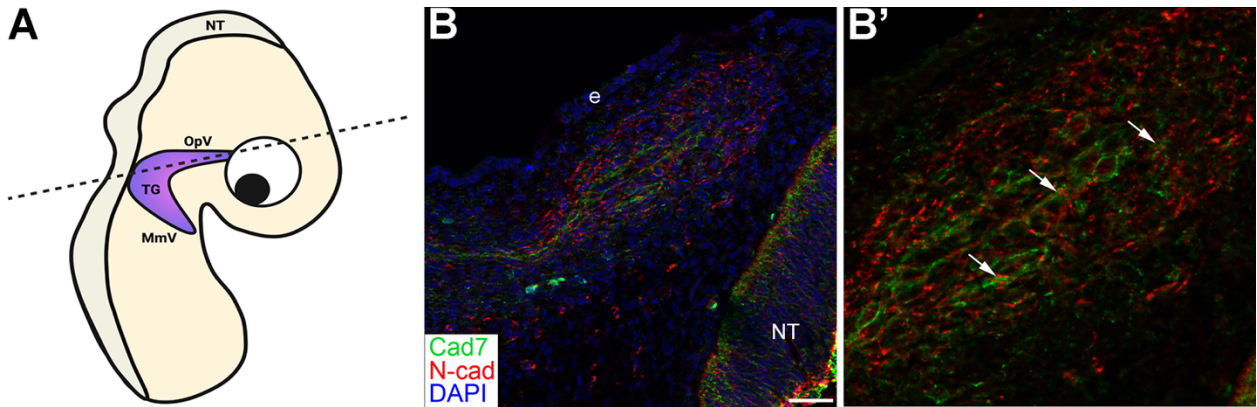


Fig. 2.10 N-cadherin and Cadherin-7 are expressed in condensing placodal neurons and neural crest cells, respectively, during chick trigeminal ganglion assembly.

(A) Cartoon diagram of forming TG in the developing chick embryo. Plane of section is shown by the dotted line. Diagram created with BioRender.com. (B) Representative transverse section taken through the forming TG followed by immunohistochemistry for Cad7 (green, marks neural crest cells) and N-cad (red, marks neurons which are all placode-derived at this stage). (C) Higher magnification (digital) of the TG in (B). DAPI (blue), cell nuclei. Scale bar in (B) is 100 μm and applies to (B') but is 50 μm . N=5 embryos. TG, trigeminal ganglion; Cad7, Cadherin-7; N-cad, neural cadherin; NT, neural tube; OpV, ophthalmic branch; MmV, maxillomandibular branch; e, ectoderm.

2.5 Discussion

Cranial neural crest cells and placode cells initially form in close proximity but become spatially separated as development ensues (Bronner & LeDouarin, 2012; Park & Saint-Jeannet, 2010; Saint-Jeannet & Moody, 2014; Theveneau et al., 2013). While these cells give rise to distinct derivatives, they will both form sensory neurons of the trigeminal ganglion, innervating much of the head and face to relay information related to pain, touch, and temperature to the central nervous system (Breau & Schneider-Maunoury, 2015; Davies, 1988; Higashiyama & Kuratani, 2014). The cellular origin of the trigeminal ganglion has been known for decades (D'Amico-Martel & Noden, 1980, 1983; Hamburger, 1961b); however, molecular mechanisms mediating early interactions between neural crest cells and placodal neurons to build the trigeminal ganglion have not been well characterized. In the chick embryo, studies uncovered the importance of cadherin-mediated interactions, as distinct cadherins are expressed by neural crest cells (Cadherin-7) (Nakagawa & Takeichi, 1995b; C. Wu & Taneyhill, 2019) and placode cells and their neuronal derivatives (N-cadherin) during early trigeminal ganglion assembly (Shiau & Bronner-Fraser, 2009a). The presence of two different cadherins on these coalescing cells begs the question as to whether heterophilic interactions exist between them to allow for proper trigeminal ganglion formation, particularly since cells expressing these cadherins can form mixed aggregates *in vitro* (Nakagawa & Takeichi, 1995b).

Our studies now address this question through the use of biochemistry and an adapted GRASP assay to examine cadherin interactions during trigeminal ganglion development. Through *in vitro* transfection experiments and use of embryonic trigeminal ganglia tissue, we demonstrate a physical interaction between Cadherin-7 and N-cadherin. This is the first report to reveal, biochemically, that Cadherin-7-N-cadherin complexes can form and, notably, are present while

the trigeminal ganglion assembles. While we cannot rule out the presence of other protein(s) in the embryo to serve as a “bridge” to allow these cadherins to associate, these data still provide strong evidence that these interactions do exist *in vivo*.

To generate the trigeminal ganglion, Cadherin-7-expressing cranial neural crest cells first migrate through the embryonic mesenchyme to the trigeminal ganglionic anlage. Here, they intermingle with newly differentiated, N-cadherin-expressing trigeminal placode-derived neurons, which have delaminated from the surface ectoderm and have also migrated through the mesenchyme. Since the cranial mesenchyme expresses N-cadherin, it is possible that the Cadherin-7-N-cadherin complexes we detected through our biochemistry studies represent interactions between Cadherin-7 on neural crest cells and N-cadherin expressed in mesenchymal cells. However, based upon the abundance of neurons in relation to the mesenchyme in dissected trigeminal ganglia, we think the primary source of N-cadherin in these interactions comes from the placodal neurons. Moreover, migratory neural crest cells form “corridors” through which placodal neurons migrate, as they provide a more permissive substrate for migration than the surrounding cranial mesenchyme (Freter et al., 2013b; Sandell et al., 2014). As such, neural crest cells and placodal neurons are tightly juxtaposed during the assembly of the trigeminal ganglion, making it more likely that the interactions we are detecting arise from Cadherin-7 on neural crest cells and N-cadherin on placodal neurons.

To further define and directly visualize these heterophilic cadherin interactions, we conducted a GRASP assay in cell culture and in the embryo. We generated two split GFP constructs (GFP domains 1-10 or GFP domain 11) fused to both Cadherin-7 and N-cadherin and examined the ability of these cadherins to associate in *cis* and in *trans* to generate GFP. Through cell culture co-transfection experiments, we demonstrated that GFP could be reconstituted as long

as the split GFP constructs were complementary, providing further evidence that Cadherin-7 and N-cadherin can interact in *cis*. Importantly, no GFP was generated after co-transfection of like split GFP moieties fused to different cadherins, pointing to the specificity of the GFP reconstitution.

Intriguingly, we noted that transfection of cells with like cadherins and complementary GRASP constructs gave rise to cells that often exhibit a round shape or are only somewhat fibroblastic, with few protrusions emanating from the cell (Fig. 5). Conversely, transfection of cells with different cadherins and complementary GRASP constructs caused cells to adopt a much more fibroblastic, and often spindly, morphology. This could be due to the presence of both a Type I (N-cadherin) and Type II (Cadherin-7) cadherin in these cells. A parallel to this can be found *in vivo* with respect to the overlapping expression domains of N-cadherin and Cadherin-7 in the developing chick spinal cord (J. Lin et al., 2014). These neuroepithelial cells are organized in a pseudostratified manner and thus exhibit a spindly morphology as they are densely packed within the neural tube/forming spinal cord.

Quantification of GFP in our transfection experiments led to the conclusion that more GFP-positive cells were present upon transfection of complementary split GFP constructs fused to the same cadherin (homophilic interactions) compared to transfection of complementary split GFP constructs fused to different cadherins (heterophilic interactions). These findings are consistent with measurements of dissociation constants in homophilic and heterophilic cadherin cell aggregates *in vitro*, which revealed that homophilic cadherin interactions are stronger than heterophilic interactions (Katsamba et al., 2009). Moreover, we find that the number of GFP-positive cells is highest in cells transfected with split GFP constructs fused to N-cadherin compared to Cadherin-7. These results are in keeping with a report showing that N-cadherin confers a much higher degree of adhesivity compared to Cadherin-7 (Chu et al., 2006b).

Finally, we noted a statistically significant 1.5-fold increase in GFP-positive cells upon transfection with Cad7 GFP1-10 + N-cad GFP11 compared to Cad7 GFP11 + N-cad GFP1-10 ($p=0.013$). This trend is also observed *in vivo* (see below). While we might expect these numbers in our heterophilic experiments to be similar, the difference could be explained by the inability of cells to effectively express N-cad GFP1-10, or, conversely, the capacity of cells to more readily express Cad7 GFP11. We favor the former idea, however, for the following reasons. Given that the N-cadherin coding sequence is larger than the Cadherin-7 coding sequence (~300 nucleotides difference), coupled with the greater size of the split GFP1-10 moiety, it is possible that cells do not readily express N-cad GFP1-10 (compared to N-cad GFP11). Further, since homophilic cadherin interactions are stronger and more stable than heterophilic ones (Katsamba et al., 2009), heterophilic interactions are, by nature, more transient. As such, the GFP we observe is a direct readout of these cadherin interactions and is correlated with their strength. Thus, we would expect homophilic interactions to yield the most GFP signal, with heterophilic interactions following next, as noted in both our *in vitro* and *in vivo* experiments. If cells do have more difficulty expressing N-cad GFP1-10, we would expect the GFP signal to be stronger in cells expressing Cad7 GFP1-10 + N-cad GFP11 versus those expressing Cad7 GFP11 + N-cad GFP1-10, which is in keeping with our results.

With these tools, we next explored the ability of Cadherin-7-expressing neural crest cells to associate with N-cadherin expressing placodal neurons. Sequential electroporation experiments were conducted in which complementary split GFP constructs were introduced into neural crest cells (Cadherin-7 split GFP construct) followed by placode cells (N-cadherin split GFP construct). Because of the anatomy of the chick embryo at the time of electroporation and tissue of origin of neural crest cells (dorsal neural folds) and placode cells (surface ectoderm), we can precisely, and

independently, target each cell type. Notably, we observed GFP fluorescence at sites where neural crest cells and placodal neurons come into contact, visualized on sections taken through the developing trigeminal ganglion. These data reveal that Cadherin-7 and N-cadherin can interact in *trans* in different cell populations, providing insight into the ability of different cadherin expressing cells to associate *in vivo*.

Although the number of GFP-positive regions was not extraordinarily high in this *in vivo* assay, this is to be expected given the nature of the electroporation, in which only a small amount of each split GFP construct was electroporated into each cell type in order to avoid potential artifacts of overexpression. Quantification of the number of GFP-positive regions/puncta through serial trigeminal ganglion sections, however, revealed results that were in keeping with what we observed *in vitro*. Specifically, we noted a statistically significant 1.6-fold increase in the GFP fluorescence reported in Cad7 GFP1-10 + N-cad GFP11-electroporated embryos vs. Cad7 GFP11 + N-cad GFP1-10-electroporated embryos ($p=0.014$). These results align with what we observe *in vitro*, with these differences also possibly arising for similar reasons as outlined above (e.g., ability of cells to effectively express N-cad GFP1-10). Alternatively, there might be inherent differences in the ability of neural crest cells and placode cells to transcribe and translate expression constructs like these, with neural crest cells more easily expressing Cad7 GFP1-10 compared to placode cells expressing N-cad GFP1-10. Finally, the heterophilic interactions we are examining occur in *trans*, not in *cis* as in our *in vitro* experiments. As such, they are dependent upon a cadherin split GFP construct getting not only electroporated but also appropriately trafficked, and correctly targeted, to a region of the plasma membrane, where it will then be in close proximity to a complementary cadherin split GFP construct on the other cell type. For these reasons, fewer heterophilic interactions may ensue in a given electroporated tissue. On the other hand, electroporation of N-

cad GFP1-10 + N-cad GFP11 into placode cells results in robust GFP fluorescence, as these electroporations are performed in *cis*.

Other pathways have been discovered to regulate cellular interactions occurring during initial chick trigeminal ganglion coalescence, including Slit1-Robo2 (Shiau et al., 2008b; Shiau & Bronner-Fraser, 2009a), Wnt (Shiau et al., 2010; Shigetani et al., 2008b), Neuropilin/Semaphorin (Gammill et al., 2007; Schwarz et al., 2008), and various growth factors (McCabe et al., 2007) (e.g., Platelet-Derived Growth Factor (McCabe & Bronner-Fraser, 2008)), with many of these also identified in the developing mouse trigeminal ganglion (Karpinski et al., 2022; Maynard et al., 2020). In chick embryos, Robo2 signaling likely modulates levels of N-cadherin post-translationally, but the mechanisms underlying this are still not well characterized. Upstream pathways regulating Cadherin-7 expression in neural crest cells also remain obscure, but it is plausible that the preceding signal transduction pathways could impact the expression of Cadherin-7 and/or N-cadherin during trigeminal gangliogenesis. Future studies aimed at addressing this question will provide important insights into the regulation of neural crest-placodal neuron migration and adhesion.

The juxtaposition of Cadherin-7-expressing neural crest cells and N-cadherin-expressing placodal neurons in the forming trigeminal ganglion hinted at the possibility that heterophilic interactions between these two cadherins could, in part, mediate this process. While the functional roles of each cadherin in trigeminal ganglion assembly have been well described, less attention was paid to the importance of their expression in building the ganglion. Cultured cells expressing either Cadherin-7 or N-cadherin can form intermingled aggregates (Nakagawa & Takeichi, 1995b), supporting the notion of heterophilic interactions and further assessed herein, but it was not evaluated *in vivo* until our studies. We now provide data uncovering a physical interaction

between Cadherin-7 in neural crest cells and N-cadherin in placodal neurons within the trigeminal ganglion. Altogether, these findings shed light on the molecular mechanisms underscoring intercellular interactions requisite for trigeminal ganglion assembly during early chick embryonic development.

Chapter 3: N-cadherin facilitates trigeminal sensory neuron outgrowth and target tissue innervation

Adapted from the following article published in the journal: *Development*

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3.1 Abstract

The trigeminal ganglion emerges from the condensation of two distinct precursor cell populations, cranial placodes and neural crest. While its dual cellular origin is well understood, the molecules underlying its formation remain relatively obscure. Trigeminal ganglion assembly is mediated, in part, by neural cadherin (N-cadherin), which is initially expressed by placodal neurons and is required for their proper coalescence with neural crest cells. Axon outgrowth first occurs from placodal neurons, but as gangliogenesis proceeds, neural crest cells also differentiate into N-cadherin-expressing neurons, and both extend axons toward targets. However, the role of N-cadherin in axon outgrowth and target innervation has not been explored. Our data show that N-cadherin knockdown in chick trigeminal placode cells decreases trigeminal ganglion size, nerve growth and target innervation *in vivo*, and reduces neurite complexity of neural crest-derived neurons *in vitro*. Furthermore, blocking N-cadherin-mediated adhesion prevents axon extension in most placodal neurons *in vitro*. Collectively, these findings reveal cell- and non-cell autonomous functions for N-cadherin, highlighting its crucial role in mediating reciprocal interactions between neural crest- and placode-derived neurons throughout trigeminal ganglion development.

3.2 Introduction

Sensory ganglia transmit various inputs from the body and environment to the central nervous system (Vermeiren et al., 2020). Within the head, the cranial sensory ganglia perform these functions for their respective cranial nerves. The trigeminal nerve, which is the largest and most complex of the cranial nerves, relays the majority of somatosensory information within the head (Heimer, 1983) through three major branches – the ophthalmic (OpV), maxillary (MxV) and mandibular (MnV) – that innervate the forehead and eye region, upper jaw, and lower jaw, respectively. The cell bodies for this nerve are positioned within the trigeminal ganglion, which arises from neural crest and placode cells (D’Amico-Martel & Noden, 1980; Hamburger, 1961b). While both cell types differentiate into trigeminal sensory neurons (Hamburger, 1961b), the neural crest also gives rise to glial cells. Trigeminal neural crest cells originate from the midbrain and anterior hindbrain (Lumsden et al., 1991; Noden, 1973) while the placodal contribution arises from the OpV and maxillomandibular (MmV) placodes. Although both of these placodes comprise the trigeminal ganglion, they differ in two important ways. First, they provide neurons to distinct lobes of the trigeminal ganglion (OpV versus MxV or MnV, respectively). Second, OpV placode cells begin exiting the cell cycle and differentiating into neurons as early as embryonic day (E)1/Hamburger-Hamilton stage (HH)8 and continue until E3.5/HH21 (McCabe et al., 2009), traveling to the trigeminal ganglion-forming region as a combination of neuroblasts and differentiated neurons. Conversely, the MmV placode generates neuroblasts that do not differentiate or become post-mitotic until they reach the ganglionic anlage (Begbie, 2002; Graham & Shimeld, 2013; McCabe et al., 2009; Shiao et al., 2011). Notably, in the chick, placodal neurons are the first neurons present within the trigeminal ganglion, differentiating and growing towards the periphery to make initial connections with their targets within 2-5 days of development. In

contrast, neural crest cells only begin neuronal differentiation at 4 days of development, and continue differentiating up to 7 days (D'Amico-Martel & Noden, 1980, 1983).

Reciprocal interactions between neural crest cells and placodal neurons are required to properly form the trigeminal ganglion (Hamburger, 1961b; Moody & Heaton, 1983; Shiau et al., 2008b). Much of this research has focused on the influence of neural crest cells on surrounding cell types, but less is known about how placode cells impact the neural crest. Because placodal neurons are present prior to neural crest-derived neurons, they may serve as pioneer neurons, providing a scaffold for later-born neurons to grow along as they innervate their targets (Bate, 1976). Although supported by some studies (Moody et al., 1989; Stainier & Gilbert, 1990), additional evidence for this is needed, and the molecules orchestrating this process remain unknown.

Intercellular adhesion among placode cells and neural crest cells regulates trigeminal gangliogenesis (Steventon et al., 2014) and may also facilitate trigeminal axon outgrowth to targets, with cadherins promoting adhesion between adjacent cells and acting as mediators of gangliogenesis (Halmi et al., 2022; Shiau & Bronner-Fraser, 2009a; Theveneau et al., 2013; C. Wu & Taneyhill, 2019). The cadherin extracellular domain facilitates *cis* and *trans* interactions via homophilic and heterophilic binding (Basu et al., 2017; Halmi et al., 2022; Straub et al., 2011), while the intracellular domain contains two binding sites for catenin proteins (p120-catenin and β -catenin) that regulate cadherin adhesive ability and provide a link to the actin cytoskeleton, respectively (Niessen et al., 2011). Neural cadherin (N-cadherin) is expressed in neural tissue at various stages of development (Hatta & Takeichi, 1986b). It is also found in mesodermal (Dady et al., 2012b; Nakagawa & Takeichi, 1995b) and ectodermal derivatives, including trigeminal and epibranchial placode cells prior to their delamination (Shiau & Bronner-Fraser, 2009a), and later

in all trigeminal sensory neurons (Inuzuka et al., 1991; Itoh et al., 1997; Redies et al., 1992; Tan et al., 2010). While multiple studies, largely in the central nervous system, describe roles for N-cadherin in regulating neurite formation (Bixby & Zhang, 1990; Gärtner et al., 2012; Hansen et al., 2008), axon fasciculation and synapse formation (Benson & Tanaka, 1998; Jontes, 2018; Riehl et al., 1996), the function of N-cadherin in sensory neurons has not been thoroughly characterized. Although N-cadherin function in placode cells is crucial for the early condensation of the chick trigeminal ganglion from undifferentiated neural crest cells and placodal neurons (Shiau & Bronner-Fraser, 2009a), whether N-cadherin mediates the later growth and maturation of all trigeminal ganglion sensory neurons, and specifically neurite production and axon extension, is unknown. Furthermore, how N-cadherin-expressing placodal neurons impact the development of later-born neural crest-derived neurons is poorly understood.

Building upon these prior studies, we demonstrate that placodal expression of N-cadherin is vital for later chick trigeminal neurodevelopment. We observed substantial deficits in trigeminal ganglion morphology and axonal projections to target tissues that arise from effects on both placode- and neural crest-derived neurons in response to N-cadherin depletion from placodal neurons. These latter defects are noted both *in vivo* and *in vitro*, providing evidence to support the hypothesis that N-cadherin-mediated adhesion is important for trigeminal neurodevelopment. Strikingly, we note decreased neurite production in neural crest-derived neurons that correlates with altered β -catenin levels, pointing to non-cell autonomous effects on the neural crest after N-cadherin knockdown in placodal neurons. These results indicate that trigeminal neurons depend on the adhesive function of N-cadherin to facilitate the outgrowth of axons. Altogether, our findings reveal a new long-term role for N-cadherin in driving the proper outgrowth of both placode- and neural crest-derived neurons throughout trigeminal gangliogenesis.

3.3 Materials and Methods

Chick embryos

Fertilized chicken eggs (*Gallus gallus*) were obtained from the Department of Animal and Avian Sciences at the University of Maryland (MD) and Centurion Poultry Incorporated (GA) and incubated at 37°C in humidified incubators on their sides. Embryos were incubated to reach desired Hamburger Hamilton (HH) stages based on times provided therein (Hamburger & Hamilton, 1992).

Morpholinos

To reduce N-cadherin protein expression, a validated lissamine-tagged translation-blocking morpholino antisense oligonucleotide (MO) against the N-cadherin transcript was used (GeneTools) (Shiau & Bronner-Fraser, 2009a), with a sequence of 5'-GCGGCGTTCCTCCGCTATCCGGCACAT-3'. For some control experiments, a 5 bp mismatch N-cadherin control MO (N-cadherin MM) was used (Shiau & Bronner-Fraser, 2009a), with a sequence of 5'-GCGcCcTTCCcCTATgCcGCACAT-3' (altered base pairs are in lowercase). Both MOs were diluted with ultrapure water and 10% sucrose [in 1 x phosphate-buffered saline (PBS)] and used at a final concentration of 500 µM.

In ovo unilateral placode electroporation

To target trigeminal placode cells prior to their delamination, a unilateral electroporation was performed at E1.75/HH10-11. The N-cadherin MO, N-cadherin MM or a combination of PiggyBacRFP, a genome incorporating vector (Chen & LoTurco, 2012) (0.819 µg/µl), with PBase, its transposase (0.912 µg/µl), was expelled on top of the ectoderm on one side of the embryo using a fine glass needle. Next, a 30-gauge needle was used to puncture a small hole through the vitelline membrane so that electrodes could lay vertically above and below the ectoderm over which the

MO or plasmid had been placed. Three 9 V pulses were delivered over 50 ms at 200 ms intervals. After electroporation, eggs were resealed with tape and parafilm, and grown at 37°C to desired stages of interest. Owing to the inherent variations that arise during normal development, even when embryos are staged identically, the contralateral side of the electroporated embryo was a more appropriate control and was used for all *in vivo* studies. Embryos were screened for successful MO electroporation based on the red fluorescence of the MO prior to collection and use in subsequent assays.

Sequential electroporation method

To target both cell populations that contribute to the trigeminal ganglion, a modified sequential electroporation (Halmi et al., 2022) was utilized. Briefly, a unilateral neural tube co-electroporation was first performed on E1.5/HH9 using a combination of PiggyBacGFP (0.819 µg/µl) and PBase (Wang et al., 2008) (0.912 µg/µl). Two, 15 V pulses were delivered over 30 ms at 200 ms intervals. The electroporated embryos were re-incubated until they reached E1.75/HH10-11, and then the placode cells on the same side of the embryo were electroporated with the N-cadherin MO, N-cadherin MM or PiggyBacRFP in the same manner as described above. After this second electroporation, eggs were resealed with tape and parafilm, and grown at 37°C to desired stages of interest.

Immunostaining

Electroporated embryos were collected at desired stages of interest and fixed in 4% paraformaldehyde (PFA) overnight at 4°C with agitation. After fixation, embryos were washed three times for 10 min each in 1 x PBS and stored in 1 x PBS at 4°C until further processing. Primary antibodies used included anti-Tubb3 (Abcam ab78078, 1:500 for section, 1:250 for wholemount), anti-GFP (Abcam ab6662, 1:250), RFP (Invitrogen MA5-15257, 1:250), anti-TrkA

(a gift from Dr Francis Lefcort, Montana State University, Bozeman, MT, USA; 1:5000), anti-TrkB (a gift from Dr Francis Lefcort, 1:2500), anti-cleaved caspase 3 (Cell Signaling Technology 9664, 1:100) and anti- β -catenin (Santa Cruz Biotechnology sc-393501, 1:100). Secondary antibodies used at 1:250 included goat anti-rabbit IgG 488 (Invitrogen A211034), goat anti-mouse IgG1 594 (Invitrogen A21125), goat anti-mouse IgG2b 647 (Invitrogen A21242), goat anti-mouse 405 (Invitrogen A31553) and goat anti-mouse IgG2a 555 or 647 (SouthernBiotech 1080-31 or 1080-32).

Tissue sections

Embryos were processed through an increasing sucrose gradient [5% and 15% sucrose (Fisher) in 1 x PBS] at 4°C either overnight or until they sank to the bottom of the glass vials in which they were stored. Next, they were processed through 7.5% gelatin (Sigma) in 15% sucrose and 20% gelatin in 1x PBS, both at 37°C, either overnight or until the embryos sank. Embryos were embedded in 20% gelatin, flash frozen using liquid nitrogen and stored at -80°C until ready for use. Embedded embryos were then cryo-sectioned at 12 μ m on charged slides (VWR, 48311-703). Slide-mounted tissue sections were stored at -20°C until ready for use. For immunohistochemistry, slides were de-gelatinized by incubation in 1 x PBS at 42°C for 15-20 min. After three 5-min washes in 1 x PBS, the slides were blocked with 10% heat-treated sheep serum (Sigma, HTSS) in 1 x PBS+0.1% Triton X-100 (Sigma, PBST) at room temperature for 1-2 h in a humidified chamber, then rinsed in PBST. Primary antibodies were diluted in PBST+5% HTSS, added to the slides, covered with a glass coverslip (Fisher) to prevent sections from drying out and incubated overnight at 4°C. The following day, the coverslips were removed, and the slides were washed four times for 30 min each in PBST. Secondary antibodies were then diluted in PBST+5% HTSS, added to the slides, covered with a glass coverslip, and incubated overnight at

4°C. The next day, slides were again washed four times for 30 min each in PBST and mounted with DAPI-fluoromount media (SouthernBiotech).

Standard whole-mount immunohistochemistry

Embryos were blocked with 10% HTSS in PBST for 2 h at room temperature. After a rinse with PBST, primary antibodies were diluted in PBST+5% HTSS and incubated overnight at 4°C with agitation. The following day, the embryos were washed four times for 30 min each in PBST. Secondary antibodies were diluted in PBST+5% HTSS and incubated overnight at 4°C with agitation. The next day, embryos were again washed four times for 30 min each in PBST and stored in 1 x PBS at 4°C until they were either imaged or cleared (see below).

Modified whole-mount immunohistochemistry for iDISCO clearing

An adapted protocol was used based on methods from (Renier et al., 2014). Fixed embryos were washed twice for 1 h each in 1 x PBS and were then taken through an increasing methanol (Pharmco) in 1 x PBS gradient [50%(1 x 1 h), 80% (1 x 1 h) and 100% (2 x 1 h)], before they were bleached overnight in 5% H₂O₂ (Sigma) in 20% DMSO (Fisher)/methanol at 4°C. The next day, samples were taken through the following series of washes: 2 x 1 h in 100% methanol, 2 x 1 h in 20%DMSO/ methanol, 1 x 1 h in 80% methanol, 1 x 1 h in 50% methanol, 2 x 1 h in 1 x PBS and 2 x 1 h in 1 x PBS+0.2% Triton X-100. Samples were incubated overnight in 20% DMSO/0.3 M glycine (Fisher) in 1 x PBS+0.2% Triton X-100 at 4°C. The next day, samples were blocked in 10% HTSS/10% DMSO/1 x PBS+0.2% Triton X-100 overnight at 4°C. Embryos were washed twice for 1 h each in 0.2% Tween-20 (Sigma) in 1 x PBS with 10 µg/ml heparin (Fisher, PTwH) at room temperature before primary antibodies were added and incubated for 72 h at 4°C. Primary antibodies were diluted in 5% DMSO/5% HTSS/PTwH. Samples were washed in PTwH for 24 h before secondary antibodies were added (diluted in the same manner as the primary antibodies),

and samples were incubated with secondary antibodies for 48 h at 4°C. Samples were then washed for 48 h in PTwH before iDISCO clearing (see below).

Explant immunocytochemistry

Explant slides were washed once with 1 x PBS, followed by fixation in 4% PFA for 1 h at room temperature with agitation. After fixation, slides were rinsed with 1 x PBS and stored in 1 x PBS at 4°C until ready for immunocytochemistry. A hydrophobic perimeter was drawn around the slides to prevent dehydration of tissue using an ImmEdge Pen (Vector Laboratories), and explants were taken through blocking, primary antibody incubation and secondary antibody incubation in the absence of coverslips, as described in the tissue section methods. Slides were mounted with DAPI-fluoromount media (SouthernBiotech) and covered with a glass coverslip.

Tissue clearing

As embryos progress through development and tissue structures surrounding the trigeminal ganglion become more complex, visualization of the trigeminal ganglion after whole-mount immunohistochemistry becomes increasingly difficult. Therefore, clearing methods to render the tissues transparent were performed. At early stages (up to E3.5/HH22), the FRUIT (Hou et al., 2015) tissue clearing protocol was used, whereas later stages required iDISCO (Renier et al., 2014) tissue clearing. With both protocols, MO fluorescence was quenched during the clearing process.

FRUIT

Following the standard whole-mount immunohistochemistry protocol, embryos were progressed through increasing concentrations of fructose (35%, 40%, 60% and 80% w/v) in 8 M urea (Sigma), 0.5% (v/v) alpha-thioglycerol (Fisher) dissolved in ultrapure water ('FRUIT buffer') for a minimum of 8 h, up to 24 h at room temperature, depending on the stage of the embryo. A sample was only moved up to the next FRUIT buffer once it had visibly changed transparency. The FRUIT

cocktail, containing increasing concentrations of fructose, was made by first dissolving fructose in ultrapure water at 37°C. Once dissolved, the urea was added, and the solution was rocked at room temperature until fully dissolved. Finally, alpha-thioglycerol was added, and buffers were stored at 4°C until use. Embryos were imaged in 60% or 80% FRUIT buffer (depending on the stage of the embryo) in glass-bottomed Petri dishes (MatTek P35G-1.5- 20-C). Typically, embryos up to E3/HH19 were imaged in 60% FRUIT while older embryos were imaged in 80% FRUIT.

iDISCO

Following the modified whole-mount immunohistochemistry protocol, embryos were washed in 1 x PBS+0.2% Triton X-100 for a minimum of 48 h. Samples were incubated in 50% (v/v) tetrahydrofuran (Sigma, THF) in water overnight in glass vials, making sure that over 90% of the vial was filled with the solution. The following day, embryos were then incubated in 80% THF in water for 1 h, followed by two 1 h incubations in 100% THF. Next, samples were incubated in 100% dichloromethane (Sigma) for 1-2 h (or until they sank to the bottom of the vial). Finally, embryos were incubated in dibenzyl ether (Sigma, DBE) overnight and imaged the following day in DBE. All steps, including embryo storage in DBE, were performed at room temperature. *iDISCO*-cleared embryos were imaged in a glass Petri dish containing a custom chamber (Renier et al., 2014), which was printed in Terrapin Works, part of the School of Engineering at the University of Maryland.

TUNEL assay

To assess apoptosis (TUNEL), the In Situ Cell Death Detection Kit, Fluorescein (Roche) was used according to manufacturer's instructions. Briefly, after secondary antibody washes (4 x 30 min in PBST), slides were washed once for 30 min in 1 x PBS. Slides were re-fixed with 4% PFA for 20 min at room temperature, followed by one 30-min wash in 1 x PBS. The TUNEL reaction mixture

was added to the slides (75 μ l per slide), and a glass coverslip was placed over them. Slides were incubated in the dark for 1 h at 37°C. Next, the coverslips were removed, and the slides were washed three times for 5 min each in 1 x PBS. Finally, slides were mounted with DAPI-fluoromount media (SouthernBiotech).

Trigeminal ganglion explant assay

Wild-type E2/HH13-14 embryos were removed from eggs and stored on ice in 1 x PBS until dissections. Trigeminal ganglia were dissected on a sylgard-coated dish by first bisecting the embryo and then carefully taking the area of the forming trigeminal ganglion, making sure to exclude the eye and neural tube. Explants were cultured on two-well glass chamber slides (ThermoFisher) coated with Poly-L-Lysine (0.01% PLL, Sigma) and fibronectin (0.01%, Sigma). To make the coated slides, PLL was added to each chamber on the glass slides and incubated inside a biosafety cabinet for 30 min at room temperature. The PLL was removed, and slides were washed three times with water. Next, fibronectin [diluted in DMEM media (ThermoFisher)] was added to each chamber and incubated at 37°C for a minimum of 2 h. The fibronectin solution was removed and slides were stored at 4°C until needed (for up to 1 week). Explants were grown in DMEM media supplemented with N2 (1%, ThermoFisher) and penicillin/streptomycin (1%, Fisher). Explants for N-cadherin function-blocking antibody experiments (see below) were grown in the supplemented DMEM media mentioned above. Sequentially labeled cells for N-cadherin MO- or MM-containing explant experiments were grown in Neurobasal media (ThermoFisher) supplemented with B27 (1%, ThermoFisher), N2 (0.5%, ThermoFisher), Glutamax (1%, ThermoFisher), penicillin/streptomycin (1%, ThermoFisher), and with nerve growth factor (ThermoFisher), brain-derived neurotrophic factor (Amgen) and neurotrophin 3 (Amgen), all at 50 ng/ml.

N-cadherin function-blocking antibody experiments

After 24 h of incubation, fresh media containing either an antibody to block N-cadherin function, MNCD2 (anti-N-cadherin, Developmental Studies Hybridoma Bank, 0.1 µg/ml) or a normal rat IgG control (Santa Cruz Biotechnology, 1.6 µg/ml) replaced the initial explant plating media, and explants were incubated overnight. The following morning, the slides were briefly rinsed with 1 x PBS and then fixed in 4% PFA for 1 h at room temperature. The explants were washed three times in 1 x PBS and then processed for immunocytochemistry.

Immunoblotting

E6.5/HH28-30 N-cadherin MO-electroporated and contralateral OpV lobes from six embryos per replicate were dissected, pooled and lysed in lysis buffer [50 mM Tris (pH 7.5), 100 mM NaCl and 0.5% IGEPAL CA-630) supplemented with cOmplete Protease Inhibitor Cocktail (Roche) and 1 mM PMSF for 30 min on ice with periodic gentle vortexing. Lysates were then centrifuged at 20,627 g for 20 min at 4°C to separate fractions and the soluble protein fraction was then quantified by Bradford assay (ThermoFisher). 12 µg of each sample was boiled at 99°C for 5 min in 4 x reducing Laemmli sample buffer. Samples were separated by 12% SDS-PAGE then transferred to a PVDF membrane (ThermoFisher). The membrane was blocked in 5% milk (in 1 x PBS and 0.1% Tween-20) for 30 min at room temperature followed by overnight incubation at 4°C with the following primary antibodies diluted in blocking solution: phosphor-histone H3 (Sigma 06570, 1:500), N-cadherin (DSHB MNCD2, 1:100) and GAPDH (GeneTex GTX627408 1:1000). Membranes were washed three times for 10 min each in 1 x PBS+0.1% Tween-20 before incubation with secondary antibodies (species- and isotype-specific horseradish peroxidase, Jackson ImmunoResearch) diluted in blocking solution for 1 h at room temperature. Membranes were washed three times for 10 min each in 1 x PBS and 0.1% Tween-20 and antibody detection

was performed using Supersignal West Pico or Femto chemiluminescent substrates (ThermoFisher) and visualized using a ChemiDox XRS system (Bio-Rad).

Quantitative PCR

Trigeminal ganglion explants in which placode cells were electroporated with PiggyBacRFP plus PBase underwent rat IgG or anti-N-cadherin treatment (all as described above). Explant cultures were pooled and lysed in RNA lysis buffer and total RNA was extracted using the RNase Mini Kit (Qiagen). cDNAs were synthesized using the SuperScript IV One-Step RT-PCR System (ThermoFisher) according to the manufacturer's instructions. Quantitative PCR was performed with iTaq Universal SYBR Green Supermix (Bio-Rad) according to the manufacturer's instructions. Equal amounts of cDNA for rat IgG- and anti-N-cadherin-treated samples were used for quadruplicate reactions, mixed with SYBR Green Supermix, and 750 nM of each primer (RFP or chick 18s rRNA). To validate results, quantitative PCR was also performed on cDNA samples prepared in the absence of reverse transcriptase and any template cDNA (no template control). RFP levels were normalized against chick 18s rRNA levels, as described previously (Schiffmacher et al., 2016). Average normalized values of RFP were plotted as bar graphs. Primer sequences are as follows: RFP forward, 5'-CTCCGAGGACGTCATCAA-3'; RFP reverse, 5'-TTGGTCACCTTCAGCTTGG-3'; chick 18s rRNA forward, 5'-TGTGCCGCTAGAGGTGAAATT-3'; and chick 18s rRNA reverse, 5'-TGGCAAATGCTTTCGCTTT-3'.

Confocal imaging

All images were acquired with the LSM Zeiss 800 confocal microscope. Laser power, gain and offset were kept consistent for the different channels during all experiments where possible. Fiji (NIH) (Schindelin et al., 2012) and Adobe Photoshop CC 2023 were used for image processing.

For wholemount imaging with z-stacks, all embryos were imaged at a 5 μm interval. For sections, images of at least five serial transverse sections were acquired and used for analysis. For all experiments, a minimum of five replicates was obtained.

Quantifications and statistical analyses

All quantifications were carried out in Fiji and all graphs were generated using Prism GraphPad Version 10.

Area measurement

Maximum intensity z-projections were generated through the trigeminal ganglion. The polygon tool was used to outline the perimeter of Tubb3 signal, and area measurements were taken for the whole trigeminal ganglion, the OpV lobe and the MmV lobe. Individual area measurements for each developmental stage grouping were averaged together and plotted to generate graphs.

Cell counting

Cell counts were either performed manually or using the Squassh (Rizk et al., 2014) plugin from the MOSAICSuite (Shivanandan et al., 2013), as described previously (Halmi et al., 2022). The Tubb3 channel of each image was used to crop the trigeminal ganglion, which was then used to crop the images for the channel of interest. For Squassh, segmentation parameters were kept uniform for all images and run as a batch function. The quality of image segmentation was confirmed visually, and adjustments were made as necessary. The number of puncta/cells was calculated per image and averaged to obtain a mean number per replicate, which was then divided by the average area of the trigeminal ganglion to adjust for size differences. Individual averages for each developmental stage grouping were plotted to generate graphs.

Nerve branching

Z-stack images of the OpV branch of the trigeminal nerve were analyzed using custom ImageJ Macro language (IJM) scripts. Maximum intensity z-projections were generated and input into the ImageJ macro. Signal profiles were obtained via successive vertical line selections using `getProfile()` at 30 μm intervals. The number of terminal nerve branches present at increasing distances from the starting point were exported and calculated in R (<https://www.R-project.org>). All scripts are available at <https://zenodo.org/records/15257825>. Data were aligned to ensure consistent starting points for each replicate, and the mean number of nerve branches was plotted to generate graphs.

Nerve width

Nerve width measurements for both the lateral and medial nasal nerves were generated using maximum intensity z-projections and the straight line tool. The width of individual OpV nerve divisions (prior to extensive branching within the target tissue) was measured based on the Tubb3 signal for each embryo. Widths for N-cadherin MO-treated embryos and their contralateral control sides were averaged and plotted to generate graphs.

Innervation area

Maximum intensity z-projections were used to generate innervation area measurements for both the lateral and medial nasal nerves. The polygon tool was used to draw a border around the Tubb3 signal in the target tissues where terminal nerve branching occurred. Area measurements for N-cadherin MO-treated embryos and their contralateral control sides were averaged and plotted to generate graphs. Intersection analysis following treatment with anti-N-cadherin Background was removed and the ridge-detection plug-in (Steger, 1998) was used to highlight axonal outgrowth and to quantify axon length from explant images. Using the detected segment binary images and the concentric circle plug-in, 20 concentric circles were overlaid on the explant, centered at the

middle. The numbers of axonal intersections at each successive ring of increasing diameter were counted and plotted to generate graphs.

RFP-labeled placode cell analysis

Background was eliminated from explant images of the Tubb3 channel and the polygon tool was used to isolate the core of the explant from its associated axonal projections. The fluorescent intensity of RFP signal was collected for both the axonal projections and the core of the explants. The mean axonal signal was divided by the mean core signal to generate a ratio. Ratios for individual treatments were averaged and compared between conditions.

Simple neurite tracer analysis

Z-stacks of GFP-positive neural crest-derived neurons were analyzed using the simple neurite tracer plug-in (Arshadi et al., 2021; Ferreira et al., 2014). Primary neurites were traced from the cell body through the entire z-stack. Neurites extending from primary neurites were added as branch points and termed secondary neurites. Any neurite branching off secondary neurites was marked as a tertiary neurite. The mean number and mean length of primary, secondary and tertiary neurites were plotted to generate graphs.

Immunoblot band intensity

Immunoblot images were processed with the Analyze Gel function. Equal boxes were drawn around the lanes of interest using the rectangle tool, and band intensities were plotted as peaks of signal. The line tool was used to isolate peaks and remove background, measuring the area under the peak to quantify band intensity for each protein. Phospho-histone H3 and N-cadherin were normalized against GAPDH and plotted to generate graphs.

β -Catenin analysis

Z-stack images of sequentially electroporated explants were processed using custom ImageJ Macro language (IJM) scripts (available at <https://zenodo.org/records/15257825>). GFP-positive neurons were identified over each slice using thresholding. The area of each identified cell was calculated using the analyze particles tool, and the mean β -catenin signal was quantified from the overlapping GFP signal in the z-stacks. Slices with insufficient β -catenin staining were excluded. Mean β -catenin signal was plotted to generate graphs.

3.4 Results

3.4.1 N-cadherin knockdown in placode cells disrupts trigeminal ganglion development

To elucidate the function of N-cadherin in placodal neurons during the formation and maturation of the trigeminal ganglion, a validated N-cadherin morpholino (Shiau & Bronner-Fraser, 2009a) (MO) was unilaterally electroporated into chick trigeminal placode cells at a single timepoint prior to their delamination. Embryos were grown to various endpoints (2.5-6.5 days post-incubation) to assess trigeminal ganglion morphology. Initial experiments with a 5 bp mismatch N-cadherin control MO (N-cadherin MM) (Shiau & Bronner-Fraser, 2009a) confirmed that the electroporation technique did not cause any morphological changes (Fig. 3.1). Once validated, the unelectroporated (contralateral) sides of N-cadherin MO-electroporated embryos were used as controls for all *in vivo* experiments to account for any developmental variation. Trigeminal ganglion size and nerve morphology were analyzed by whole-mount immunohistochemistry using an antibody against tubulin β 3, class III (Tubb3) to label neurons, followed by tissue clearing for imaging. Given the time difference in neuronal differentiation in the trigeminal ganglion (D'Amico-Martel & Noden, 1983; Moody et al., 1989), the Tubb3

antibody was presumed to identify placode-derived neurons up to E4/HH23, after which both placode- and neural crest-derived neurons were labeled indistinguishably.

During initial gangliogenesis (E2.5/HH16-18), N-cadherin depletion from trigeminal placode cells caused neuron dispersal, increasing the forming trigeminal ganglion area compared to the contralateral control (Fig. 3.2A,A',F), consistent with previous work (Shiau & Bronner-Fraser, 2009a). However, by E3.5/HH19-22, the size of the trigeminal ganglion on the electroporated side was significantly reduced compared to the contralateral control (Fig. 3.2B,B',F). This reduction persisted even as neural crest cells began differentiating into neurons at E4.5/HH23-25 (Fig. 3.2C,C',F), pointing to potential effects on both placode- and neural crest-derived neurons during trigeminal neurodevelopment. At both E5.5/ HH26-27 and E6.5/HH28-30, N-cadherin MO-electroporated trigeminal ganglia were no longer statistically different from controls (Fig. 3.2D-E',F).

Next, we measured the area of OpV and MmV individually. Interestingly, N-cadherin knockdown led to a significant increase in MmV, but not OpV, at E2.5/HH16-18 (Fig. 3.2G,H). However, from E3.5-E6.5/HH19-30, N-cadherin depletion did not affect MmV lobe development (Fig. 3.2H) but led to a significant reduction in OpV lobe size (Fig. 3.2D,D',G). By E6.5/HH28-30, the size of both lobes was similar to controls (Fig. 3.2G,H). We did not observe any increased cell proliferation in N-cadherin MO OpV lobes at E6.5/HH28-30, as indicated by phospho-histone H3 expression (Fig. 3.3), that would explain the absence of phenotype observed at this stage.

To investigate whether specific subpopulations of trigeminal sensory neurons were being impacted after N-cadherin knockdown, we analyzed TrkA and TrkB receptor expression in OpV sections at E5.5/HH26-27, when both placode- and neural crest-derived neurons are present in the trigeminal ganglion, as this stage grouping includes the process of neural crest cell differentiation

into neurons (D'Amico-Martel & Noden, 1983; R. Williams et al., 1995). TrkA and TrkB neurons were counted on immunolabeled sections from contralateral (Fig. 3.4A-B') and N-cadherin MO (Fig. 3.4C-D') trigeminal ganglia. N-cadherin MO treatment caused a significant decrease in TrkB-positive neurons (Fig. 3.4E); however, after normalization against the smaller area of the OpV lobe, the TrkB neuron count was no longer statistically different from controls (Fig. 3.4F). No changes were observed in TrkA-positive neurons (Fig. 3.4E,F). These results suggest that the morphological deficits observed in the trigeminal ganglion over time cannot be attributed to alterations in specific neuronal subpopulations.

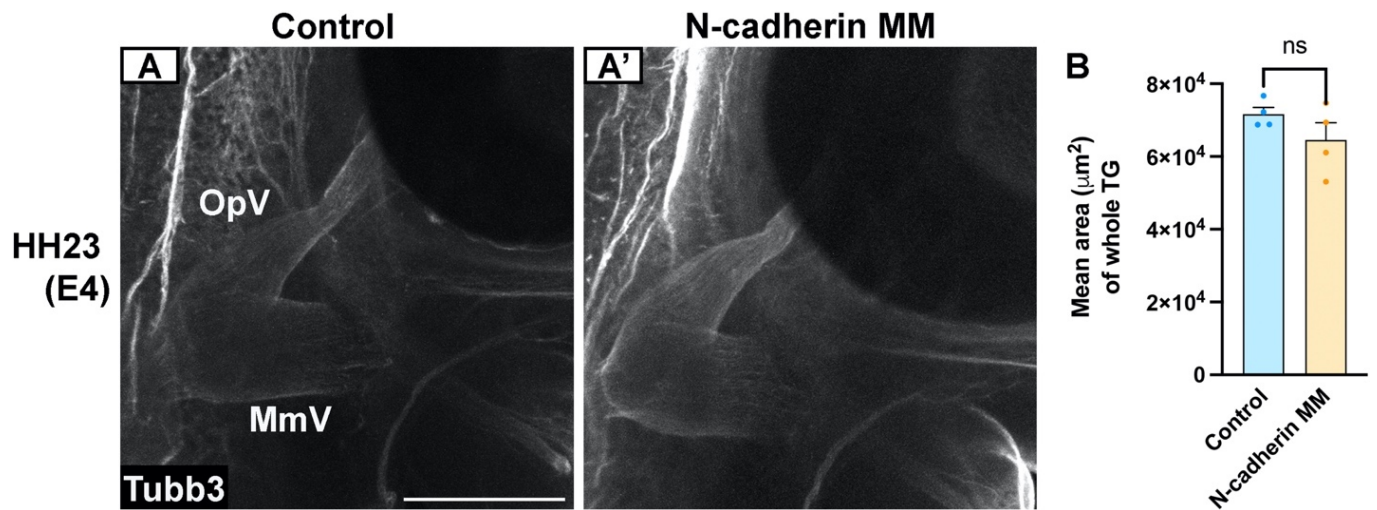


Fig. 3.1 N-cadherin mismatch control MO (MM)-treated embryos show normal trigeminal ganglion morphology.

(A-A') Representative images of the trigeminal ganglion after Tubb3 immunostaining (E4.5/HH23-25, (n=4)). Scale bar in (A) is 250 μm and applies to (A'). (B) Mean whole trigeminal ganglia area ± SEM for contralateral control and N-cadherin MM sides of embryos. Statistical significance was determined via an unpaired t-test. Abbreviations: TG: trigeminal ganglion; OpV: ophthalmic; MmV: maxillomandibular; MM: mismatch control; MO: morpholino; ns: not significant.

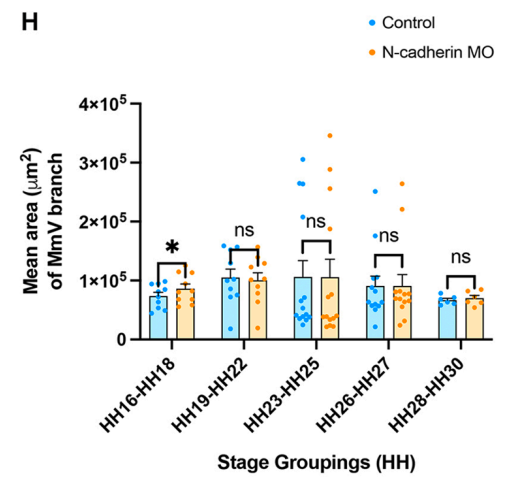
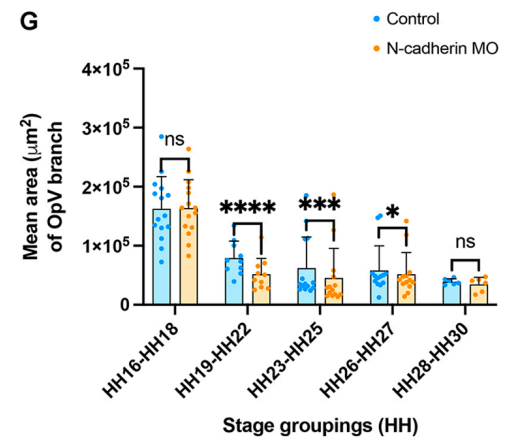
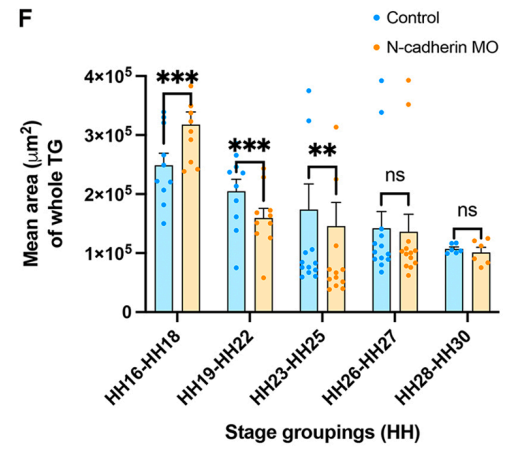
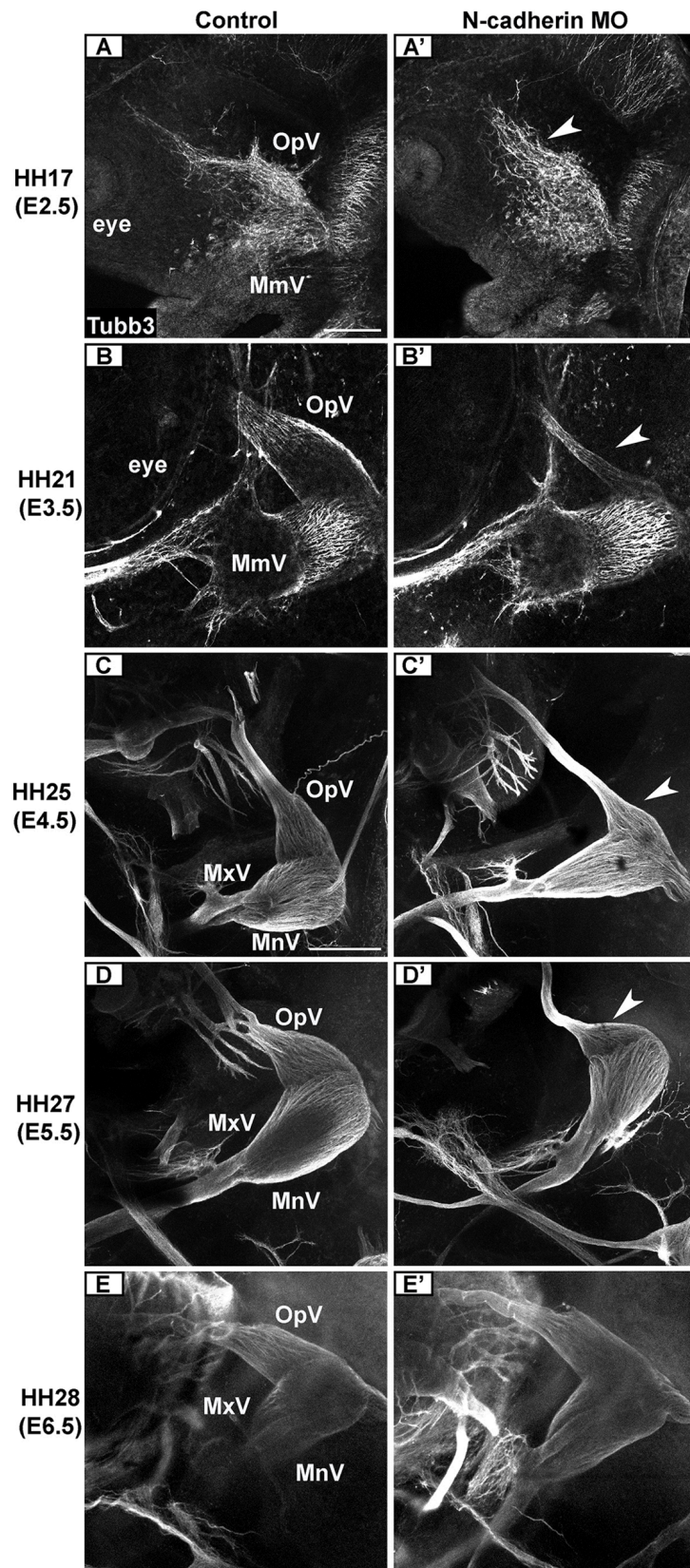


Fig. 3.2 Knockdown of N-cadherin in placode cells disrupts trigeminal ganglion development.

(A-E') Representative images of the trigeminal ganglion (contralateral control and N-cadherin MO-electroporated side) after Tubb3 immunostaining [E2.5/HH16-18: A,A' (n=10); E3.5/HH19-22: B,B' (n=10); E4.5/HH23-25: C,C' (n=14); E5.5/HH26-27: D,D' (n=13); E6.5/HH28-30: E,E' (n=6)]. Arrowheads indicate reductions in OpV lobe size in N-cadherin MO samples compared to controls. Scale bars: 250 μ m (bar in A applies to in A-B'; bar in C applies to C-E'). (F-H) Mean area $\pm$ s.e.m. of whole trigeminal ganglia (F), the OpV branch (G) and the MmV/MxV branch (H) for contralateral and N-cadherin MO sides of embryos. Statistical significance was determined using paired t-tests and the Wilcoxon signed-rank test. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001; ns, not significant. TG, trigeminal ganglion; OpV, ophthalmic; MmV, maxillomandibular; MxV, maxillary; MnV, mandibular.

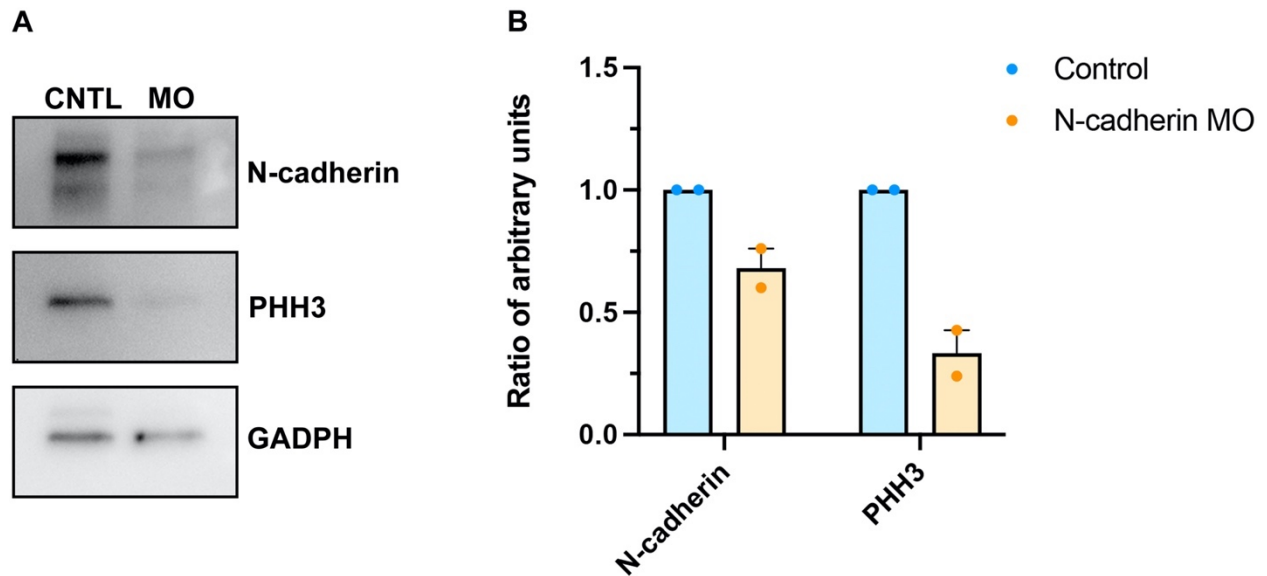


Fig. 3.3 N-cadherin knockdown in placode cells does not increase cell proliferation at later developmental stages.

(A) Immunoblots of E6.5/HH28-30 electroporated (MO) and contralateral (CNTL) trigeminal OpV lobes for N-cadherin, phospho-histone H3 (PHH3), and GAPDH (n=2). (B) Protein levels of N-cadherin and phospho-histone H3 normalized against GAPDH loading control, presented as a fraction of total signal from contralateral control OpV lobes.

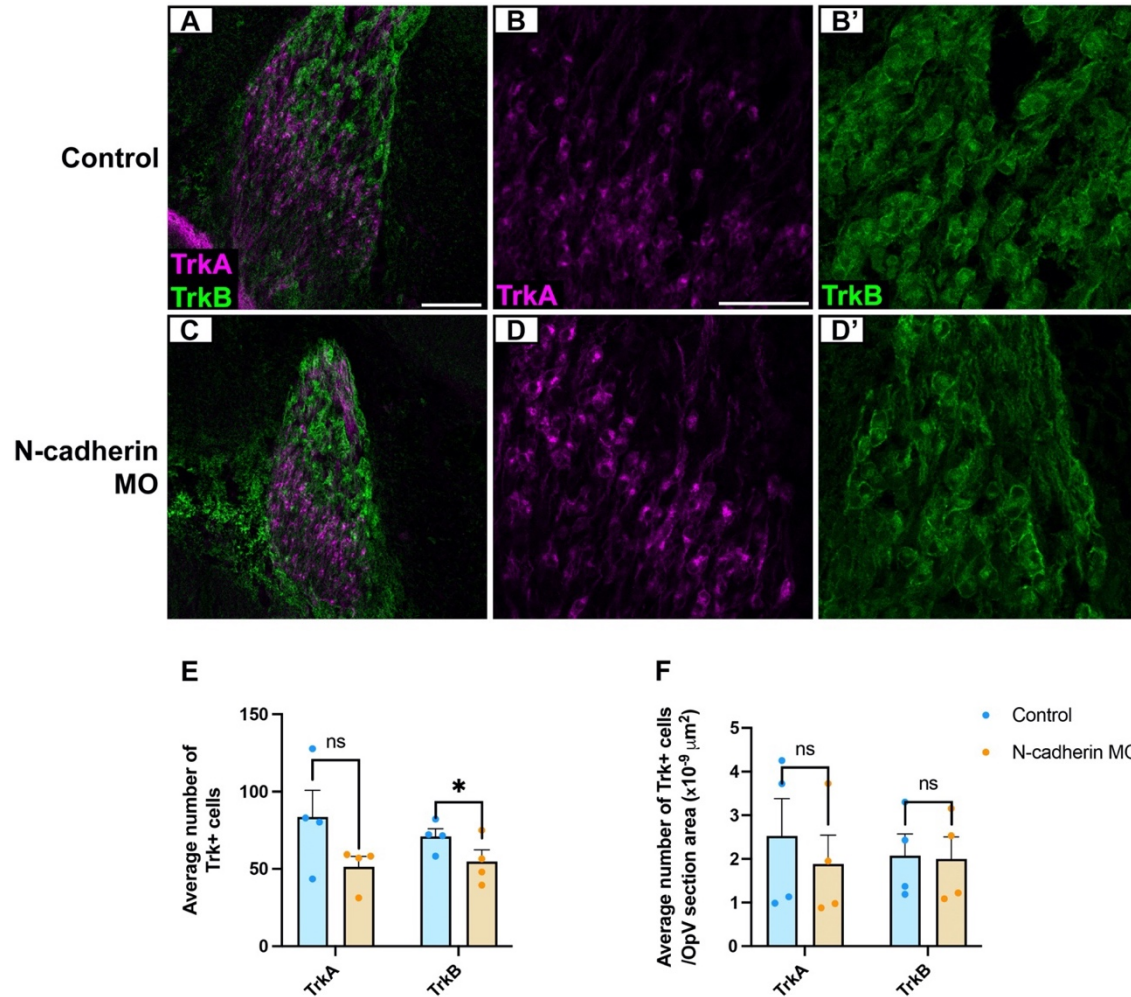


Fig. 3.4 N-cadherin depletion from placode cells does not alter subpopulations of sensory neurons within the trigeminal ganglion.

(A-D') Sagittal serial sections through the OpV lobe of the trigeminal ganglion (contralateral control (A-B') and N-cadherin MO-electroporated side (C-D')) from representative E5.5/HH26-27 (n=4) embryos with TrkA and TrkB immunostaining. Images of TrkA and TrkB serial sections were merged to generate representative images. Scale bar in (A) is 100 μm and applies to (C); scale bar in (B) is 50 μm and applies to (B, D-D'). (E) Average number of TrkA- or TrkB- positive OpV neurons $\pm$ SEM after N-cadherin MO electroporation. (F) Average number of TrkA- or TrkB-positive OpV neurons normalized against OpV lobe area $\pm$ SEM. Statistical significance was determined via paired t-tests (E, F). (*) $p < 0.05$.

3.4.2 Depletion of N-cadherin from trigeminal placode cells disrupts target innervation by OpV nerves

Since extensive morphology changes were observed within the OpV lobe (Fig. 3.2G,H), we focused subsequent *in vivo* analyses on its development and next evaluated whether OpV axons and nerve projections into cranial target tissues were also affected. We first examined the lateral nasal branch at E4.5/HH23-25, as it becomes a prominent structure within the embryonic head by this stage (Higashiyama & Kuratani, 2014). We performed wholemount Tubb3 immunohistochemistry, which labels both placode and neural crest-derived neurons at this stage, and compared the N-cadherin MO-electroporated side of the embryo to the contralateral control side. These experiments revealed fewer lateral nasal nerve branches on the N-cadherin MO side of the embryo (Fig. 3.5A,B). To quantify these findings, we assessed the distribution of terminal nerve endings at 30 μ m intervals, starting at the first axon branching point from the OpV lobe. No difference in the number of terminal nerve endings was observed closer to the trigeminal ganglion, but fewer were present in distal regions after N-cadherin MO treatment (Fig. 3.5C). Moreover, nerve width and innervation area were significantly reduced after N-cadherin knockdown when compared to contralateral control trigeminal ganglia (Fig. 3.5D,E).

Using the same method, we evaluated the medial nasal nerve at E5.5/HH26-27, as this stage provided more consistent morphological development compared to E4.5/HH23-25 embryos (Higashiyama & Kuratani, 2014), where increased variability was observed. While the number of medial nasal terminal nerve endings was similar between conditions (Fig. 3.5F-H), the nerve width and target innervation area were significantly smaller after N-cadherin knockdown compared to contralateral control trigeminal ganglia (Fig. 3.5I, J). Together, these findings reveal that N-

cadherin is required by OpV trigeminal neurons to send axons to targets, thus leading to proper nerve outgrowth, fasciculation and target innervation.

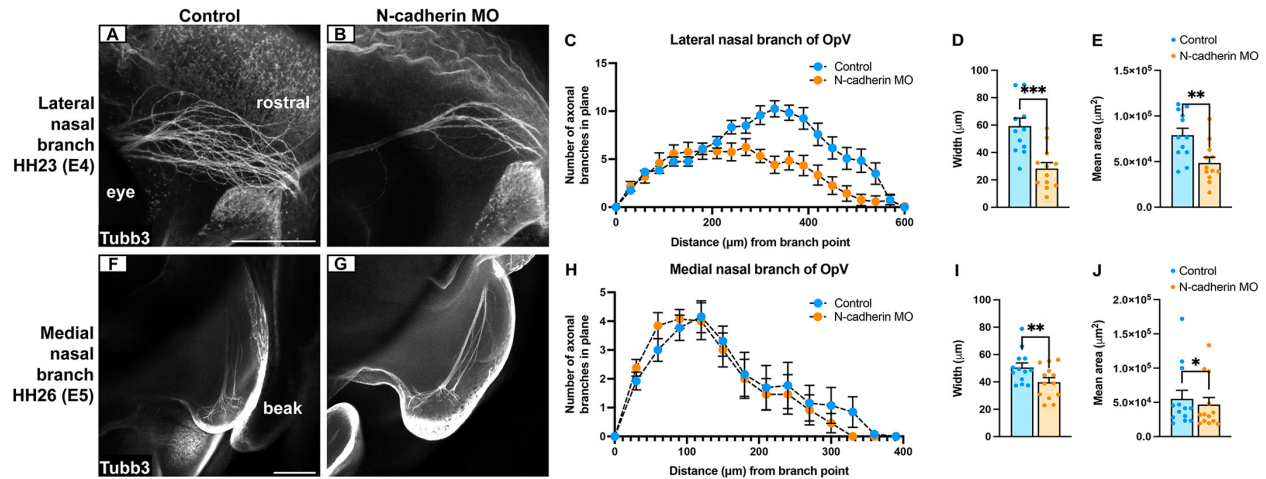


Fig. 3.5 N-cadherin depletion from placode cells negatively impacts outgrowth and target tissue innervation for OpV nerve branches.

(A,B,F,G) Representative images of the lateral (A,B) and medial (F,G) nasal nerves (contralateral control and N-cadherin MO-electroporated side) after Tubb3 immunostaining [A,B: E4.5/HH23-25 (n=12); F,G: E5.5/HH26-27 (n=13)]. Scale bars: 250 μ m (bar in A applies to A and B; bar in F applies to F and G). (C,H) Number of terminal nerve endings in the lateral (C) and medial (H) nasal branches of the OpV lobe. Data are mean $\pm$ s.e.m. Significant distances in C are observed at 420, 450, 480 and 530 μ m ($P<0.05$); at 300 and 570 μ m ($P<0.01$); and at 330, 360 and 390 μ m ($P<0.001$). No significant differences were observed in H. (D,I) Mean nerve width $\pm$ s.e.m. in control and N-cadherin MO-treated nerves. (E,J) Mean innervation area $\pm$ s.e.m. in control and N-cadherin MO-treated nerves. Statistical significance was determined using paired t-tests, with adjustments made for multiple comparisons in C,H. * $P<0.05$, ** $P<0.01$, *** $P<0.001$. Contributions from A. McIntosh.

3.4.3 N-cadherin knockdown in placode cells leads to increased cell death within the OpV lobe

To assess whether cell death altered OpV lobe size, axon outgrowth and target innervation after N-cadherin knockdown in placode cells, we performed staining for apoptotic cells using cleaved caspase 3 and TUNEL (Fig. 3.6). We examined timepoints encompassing when placode cells are first delaminating (E1.5/HH13-15) and when morphological changes were observed (Fig. 3.2: E2.5/HH16-18, E3.5/HH19-22 and E4.5/HH23-25). Apoptosis was evident in tissue sections from unelectroporated, contralateral control (Fig. 3.6A,A') and N-cadherin MO-electroporated (Fig. 3.6B,B') trigeminal ganglia. When correcting for size differences in the ganglion after N-cadherin knockdown, cell death was significantly increased on the N-cadherin MO-treated side only during initial coalescence of placodal neurons with undifferentiated neural crest cells (E2.5/HH16-18), when OpV size remained unchanged (Fig. 3.2A,A',G). Once placodal neurons and neural crest cells have formed the trigeminal ganglion, and neural crest cells have begun differentiating into neurons beyond E4, there was no significant difference in cell death between the N-cadherin MO-treated and contralateral control sides (Fig. 3.6C), despite reduced OpV size at these stages after N-cadherin knockdown (Fig. 3.2B-D',G). These data suggest that apoptosis could partially account for some of the morphological phenotypes observed during early gangliogenesis prior to neural crest cell differentiation into trigeminal sensory neurons.

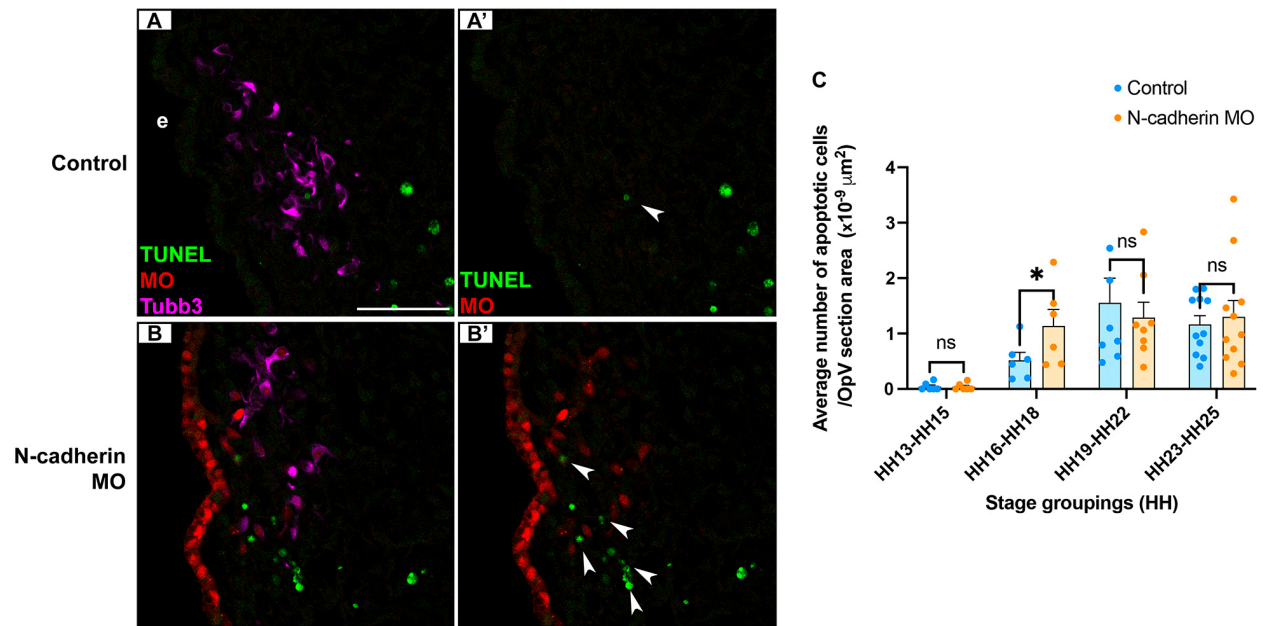


Fig. 3.6 Morphological changes in the trigeminal ganglion after N-cadherin knockdown in placode cells are in part due to increased cell death.

(A-B') Transverse sections through the OpV lobe (contralateral control and N-cadherin MO-electroporated side) from representative E2.25/HH16 embryos with Tubb3 immunostaining and TUNEL staining. Scale bar: 50 μm . (C) Five serial sections per embryo from stage groupings E1.5/HH13-16 (n=6), E2.5/HH16-18 (n=6), E3.5/HH19-22 (n=8) and E4.5/HH23-25 (n=11) were imaged and used for analysis. Arrowheads in A' and B' indicate TUNEL puncta. Statistical significance was determined using paired t-tests and Wilcoxon signed-rank test. *P<0.05; ns, not significant. e, ectoderm.

3.4.4 Neurite complexity in neural crest-derived neurons is reduced after N-cadherin knockdown in placode cells

The absence of persistent apoptosis and presence of continued axon outgrowth deficits, particularly at stages when neural crest cells differentiate into neurons, led us to hypothesize that depletion of N-cadherin from placode cells may be impacting later-born neural crest-derived neurons. To test this, we performed a sequential electroporation (Halmi et al., 2022) using a genome-incorporating vector (PiggyBacGFP) and its accompanying transposase (PBase) (Lu et al., 2009; Macdonald et al., 2012) to first label neural crest cells with a stably expressed green fluorescent protein (GFP), followed by electroporation of placode cells with N-cadherin MO or the validated N-cadherin mismatch (MM) control morpholino (Fig. 3.1), which was used as the control for this experiment (Shiau & Bronner-Fraser, 2009a). Sequential electroporations of neural crest cells and placode cells leads to mutually exclusive expression of the introduced plasmid/morpholino (Fig. 3.7), as shown previously (Halmi et al., 2022). Sequentially labeled trigeminal ganglia were dissected, cultured as explants, grown *in vitro* for 3 days, and subsequently processed for immunostaining with GFP and Tubb3 antibodies (Fig. 3.8A). Although N-cadherin MM-treated explant cultures exhibited numerous axons extending from neural crest-derived neurons (Fig. 3.9A,A'), qualitatively we observed relatively few GFP-positive neural crest-derived axons in N-cadherin MO-treated samples (Fig. 3.9B,B'). Interestingly, neurites, which precede axon specification and extension (Sainath & Gallo, 2015), were noted from GFP-positive neural crest-derived somata in both N-cadherin MO- and N-cadherin MM-treated explant cultures. These were numerous in N-cadherin MM-treated explant cultures (Fig. 3.8B,C), but noticeably fewer in N-cadherin MO-treated explants (Fig. 3.8D,E). In addition to a significant decrease in the number of primary and secondary neurites in neural crest-derived neurons, tertiary neurites were absent in

N-cadherin MO-treated samples but present in N-cadherin MM-treated explants (Fig. 3.8F). Although neurite branching was deficient in N-cadherin MO-treated explants cultures, there was no difference in the length of primary and secondary neurites compared to N-cadherin MM-treated explant cultures (Fig. 3.8G). These results indicate that N-cadherin in placodal neurons non-cell autonomously facilitate axon extension and branching in neural crest-derived neurons *in vitro*, providing an additional explanation for the sustained morphological deficits noted in the trigeminal ganglion *in vivo*.

To explore a potential mechanism behind this observation, we examined β -catenin within neural crest-derived neurons in this same assay, given its known interactions with N-cadherin and role in neurite outgrowth (Benson & Tanaka, 1998; Halbleib & Nelson, 2006b; Miyamoto et al., 2015). While membranous β -catenin was evident in neural crest-derived neurons of N-cadherin MM-treated samples (Fig. 3.8H,H', arrowheads), β -catenin levels were significantly reduced in neural crest-derived neurons of N-cadherin MO-treated samples, with less β -catenin evident along cell membranes (Fig. 3.8I,J). These findings support a mechanism whereby changes to β -catenin levels could contribute to the defects observed in neural crest-derived neurite production after N-cadherin knockdown in placode cells.

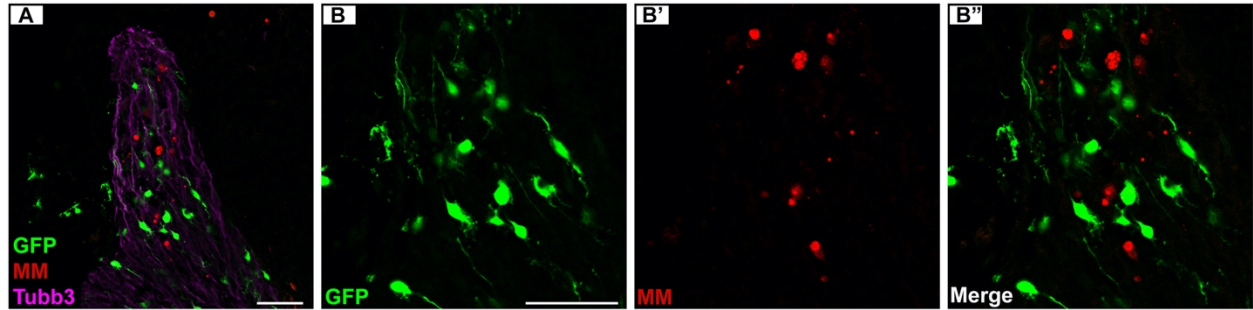


Fig. 3.7 Sequential electroporation of neural crest cells and placode cells exclusively labels distinct cell populations.

Representative images of an HH25/E4.5 embryo sequentially electroporated with PiggyBacGFP plus PBase (neural crest cells) and N-cadherin MM (placode cells), followed by Tubb3 and GFP immunostaining. Scale bar in (A) is 100 μm and scale bar in (B) is 50 μm and applies to (B'-B'').

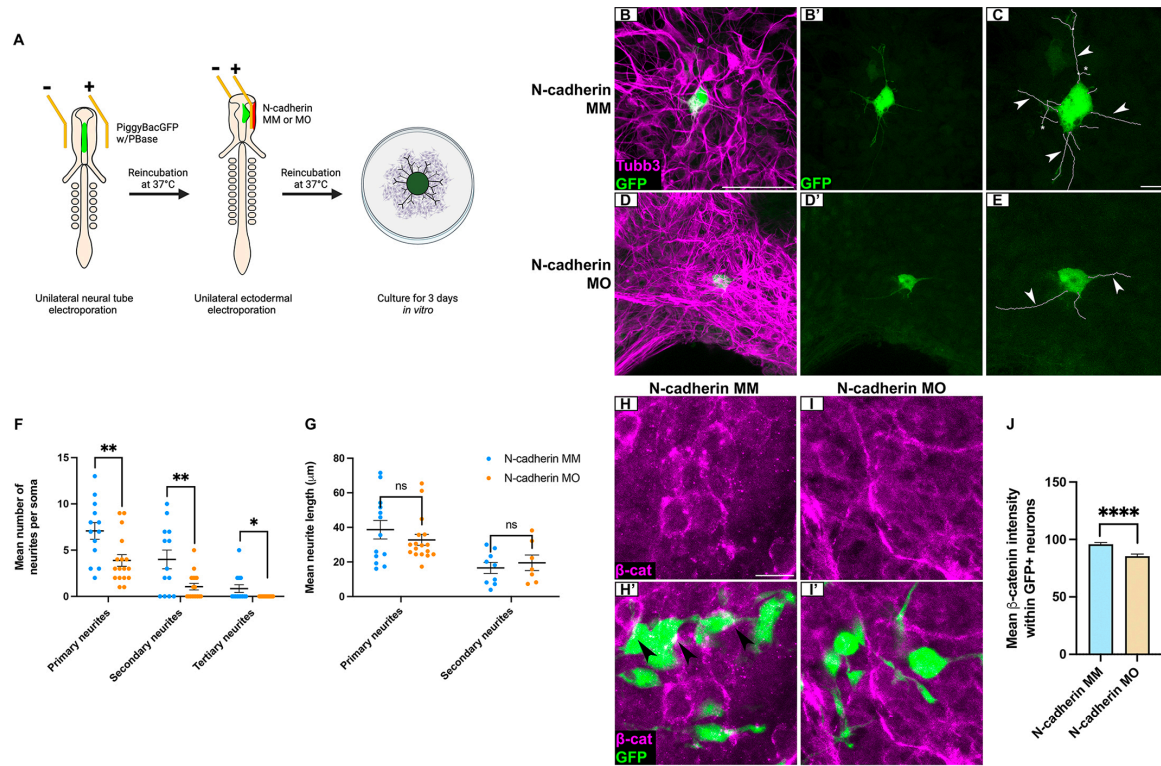


Fig. 3.8 N-cadherin depletion from placode cells decreases neurite complexity in neural-crest derived neurons.

(A) Schematic of the sequential electroporation explant culture technique. Created in BioRender. McIntosh, A. (2025) <https://BioRender.com/zj7afjj>. This figure was sublicensed under CC-BY 4.0 terms. (B-E) Representative maximum intensity z-projections of GFP-labeled neural crest-derived neurons after placodal electroporation with N-cadherin MM (B, B', control, n=13) or N-cadherin MO (D, D', n=17) from trigeminal ganglion explants following GFP and Tubb3 immunostaining, and neurite-traced images (C, E). Arrowheads and asterisks indicate examples of primary and secondary neurites, respectively. Scale bars: 50 μ m in B for B, B', D, D'; 10 μ m in C for C, E. (F, G) Number (F) and length (G) of GFP-positive neural crest-derived neurites after N-cadherin MM or N-cadherin MO electroporation. Data are mean $\pm$ s.e.m. (H-I') Representative maximum z-projections of GFP-labeled neural crest-derived neurons after placodal electroporation with N-cadherin MM (H, n=3) or N-cadherin MO (I, n=4) from trigeminal ganglion explants following

GFP and β -catenin immunostaining. Arrowheads indicate colocalization of GFP and β -catenin staining. Scale bar: 10 μ m. (J) Mean β -catenin signal intensity ($\pm$ s.e.m.) in GFP-positive neural crest-derived neurons. Statistical significance was determined using unpaired t-tests and Mann–Whitney tests. * P <0.05, ** P <0.01, **** P <0.0001; ns, not significant. Contributions from A. McIntosh.

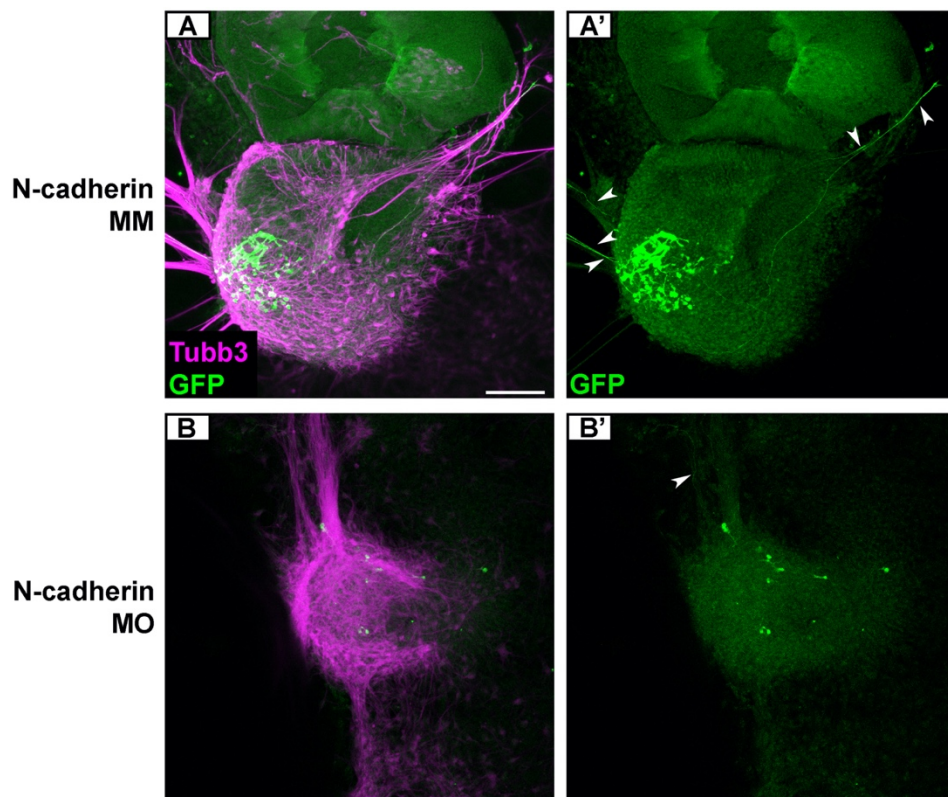


Fig. 3.9 N-cadherin MO-treated explants qualitatively exhibit less neural crest-derived axon outgrowth.

(A-B') Representative images of sequentially-electroporated trigeminal explants with neural crest cells co-electroporated with PiggyBacGFP plus PBase, and placode cells electroporated with N-cadherin MM (A-A') or N-cadherin MO (B-B'), followed by GFP and Tubb3 immunostaining.

Arrowheads in (A') and (B') point to neural crest-derived axons. Scale bar in (A) is 100 μm and applies to all images.

3.4.5 N-cadherin controls proper axon outgrowth of trigeminal neurons *in vitro*

The morphological defects observed after N-cadherin knockdown in placode cells *in vivo* and *in vitro* suggest a role for N-cadherin-mediated adhesion in facilitating axon outgrowth of both placode and neural crest-derived trigeminal sensory neurons. To test this, we used a validated function-blocking antibody against the extracellular domain of N-cadherin, known as MNCD2 (Bronner-Fraser et al., 1992; Redies et al., 1992) (anti-N-cadherin), to block N-cadherin-mediated adhesion in explanted trigeminal ganglion tissue.

Trigeminal ganglion explant cultures were grown *in vitro* for 24 h, followed by treatment with anti-N-cadherin or a control rat IgG antibody for an additional 24 h (Fig. 3.10A), and then processed for Tubb3 immunostaining (Fig. 3.10B,C,D). Wild-type explants incubated *in vitro* for 24 h without treatment served as controls ('pre-treatment') to evaluate baseline axon outgrowth and the possibility of axon pruning prior to antibody treatment. Ridge detection was used to detect segments, which were used as a proxy for individual axons (Fig. 3.10B',C',D'), and an intersection analysis was run to measure changes to axon outgrowth and branching (Fig. 3.10B'',C'',D''). While the Tubb3 fluorescence intensity between pre-treatment explants and anti-N-cadherin-treated samples was similar, a 60% reduction in Tubb3 fluorescence was observed in anti-N-cadherin-treated samples compared to rat IgG-treated samples (Fig. 3.10E). These results point to decreased axon density in trigeminal ganglion explant cultures after blocking N-cadherin based adhesion. Furthermore, intersection analyses revealed that explants treated with anti-N-cadherin exhibited a significant increase in axonal branching compared to pre-treatment samples up to 1400 μm , while rat IgG-treated explants exhibited a significant increase in branching up to 1750 μm compared to

the pre-treatment group (Fig. 3.10F). However, when comparing the anti-N-cadherin and rat IgG-treated explants, significant differences were only observed up to 700 μm (Fig. 3.10F).

Interestingly, of the neurons that did extend axons, the mean segment length of detected axons was similar between pre-treatment and anti-N-cadherin-treated explants (Fig. 3.10G), suggesting that some axons can extend in the absence of N-cadherin-mediated adhesion. However, the rat IgG-treated group had a significantly decreased average segment length compared to both, providing further support for increased axon branching over time. To verify this and confirm that the decreased segment length was not due to axon pruning, we visualized all detected segments using a violin plot to assess the distribution of the data (Fig. 3.10H). This analysis confirmed an increase in smaller segments in the rat IgG-treated group, validating that the observed decrease in mean segment length (Fig. 3.10G) resulted from averaging long and short segments, rather than from axons retracting over time. Collectively, these data suggest N-cadherin-mediated adhesion promotes axonal outgrowth and complexity in trigeminal neurons *in vitro* but likely does not function in axon trimming.

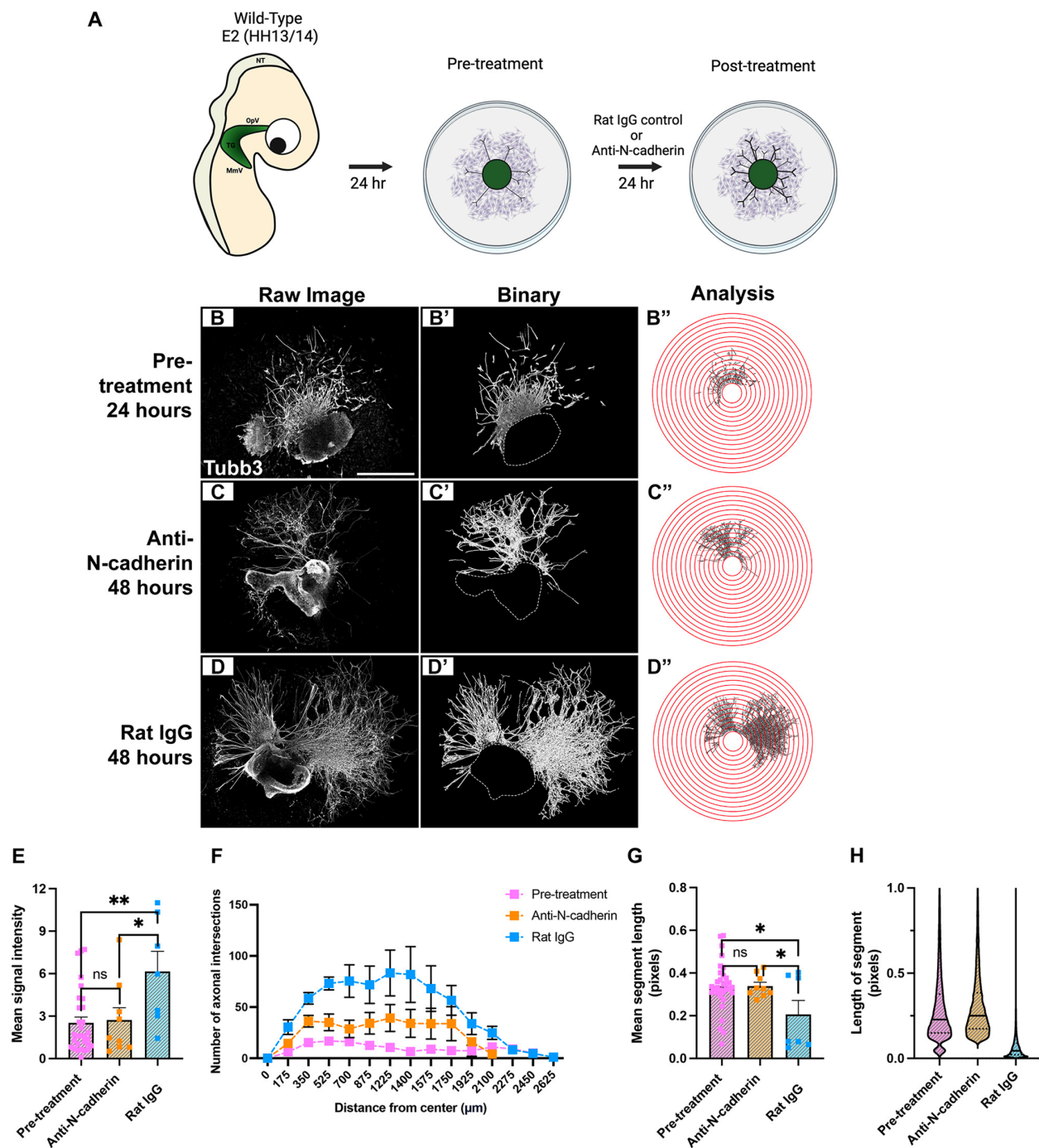


Fig. 3.10 Blocking N-cadherin-mediated adhesion reduces trigeminal neuron axon outgrowth.

(A) Schematic of a N-cadherin function-blocking assay. Created in BioRender. McIntosh, A. (2025) <https://BioRender.com/4yud5sc>. This figure was sublicensed under CC-BY 4.0 terms. (B-D'') Workflow for *in vitro* analyses. (B,C,D) Representative images of wild-type trigeminal

ganglion explants after 24 h of incubation and fixation (pre-treatment control explants, n=31), or post-treatment with either anti-N-cadherin (C, n=9) or rat IgG (D, n=7) antibody, followed by Tubb3 immunostaining. Scale bar: 1 mm. (B',C',D') Binary images generated after ridge detection processing on Fiji for pre-treatment (B'), anti-N-cadherin-treated (C') or rat IgG-treated (D') explants. Dotted lines indicate perimeter of explanted tissue cropped during processing. (B'',C'',D'') Processed images overlaid with concentric circle function to count the number of axonal intersections at increasing intervals of 175 μ m. (E) Mean signal intensity for explant outgrowth $\pm$ s.e.m. (F) Mean number of axonal intersections $\pm$ s.e.m. Significant differences between pre-treatment control explants and anti-N-cadherin-treated explants are observed at 525 μ m ($P<0.05$), and 175, 350, 875, 1225 and 1400 μ m ($P<0.01$). Significant differences between pre-treatment controls and rat IgG-treated explants are observed at 1750 μ m ($P<0.05$), 1575 μ m ($P<0.01$), and 175-1400 μ m ($P<0.0001$). Significant differences between anti-N-cadherin- and rat IgG-treated explants are observed at 175, 350 and 700 μ m ($P<0.05$), and 525 μ m ($P<0.01$). (G) Mean segment length $\pm$ s.e.m. for pre-treatment, anti-N-cadherin-treated and rat IgG-treated explants. (H) Violin plots of segment lengths for pre-treatment, anti-N cadherin-treated and rat IgG-treated explants. Solid black lines indicate median; dotted black lines indicate the interquartile range. Statistical significance was determined using unpaired t-tests and Mann–Whitney tests (E–G) with adjustments made for multiple comparisons (F). * $P<0.05$, ** $P<0.01$. Contributions from A. McIntosh.

3.4.6 N-cadherin is required cell autonomously for the axon outgrowth of the majority of placodal neurons

Our preceding findings indicate that most trigeminal sensory neurons rely on N-cadherin for axon outgrowth. To investigate their cellular origin, an ectodermal co-electroporation with a stably expressed red fluorescent protein (PiggyBacRFP plus PBase) (Lu et al., 2009; Macdonald et al., 2012) was performed to label placode-derived neurons. Next, RFP-positive explant cultures were treated with either rat IgG or anti-N-cadherin, as previously described. Following immunostaining with Tubb3 and RFP antibodies, robust axon outgrowth was observed, the majority of which overlapped with RFP-positive placode-derived neurons after rat IgG treatment (Fig. 3.11A). Many Tubb3-positive projections were also RFP positive in these control explant cultures (Fig. 3.11B-B"). Explant cultures treated with anti-N-cadherin, however, exhibited reduced overall axon outgrowth and fewer RFP-positive projections (Fig. 3.11C), as noted previously (Fig. 3.10C). While colocalization of Tubb3 and RFP was maintained in the anti-N-cadherin-treated axonal projections, the total amount of outgrowth was less (Fig. 3.11D-D"). Importantly, although overall Tubb3 fluorescence was significantly reduced in the anti-N-cadherin-treated explant cultures, reflecting fewer axons observed after blocking N-cadherin-mediated adhesion, there was no change in RFP protein (fluorescence, Fig. 3.11E) or RFP transcript (Fig. 3.11F) levels between the treatments. These findings reveal that blocking N-cadherin-mediated adhesion did not affect the presence of placodal neurons, but rather impacted axon outgrowth.

Given these data and our previous results pointing to a role for N-cadherin in axon outgrowth, we hypothesized that anti-N-cadherin-treated explant cultures would show a greater amount of RFP signal within the core of the explanted tissue, which contains the majority of the

neuronal cell bodies, due to the increased presence of placodal neurons unable to extend axons in the absence of N-cadherin-mediated adhesion. To address this, we evaluated the RFP signal found in axonal projections versus RFP signal remaining within the core of the explanted tissue (Fig. 3.11G). While 33% of the total RFP signal came from axonal projections in rat IgG-treated explants, this dropped to 15% in anti-N-cadherin-treated explants. In contrast, 67% of the total RFP signal was found in the core of the rat IgG-treated explants versus 85% in anti- N-cadherin-treated explants (Fig. 3.11H). Taken together, these data highlight a cell-autonomous requirement for N-cadherin-mediated adhesion in the proper axon outgrowth of placode-derived neurons.

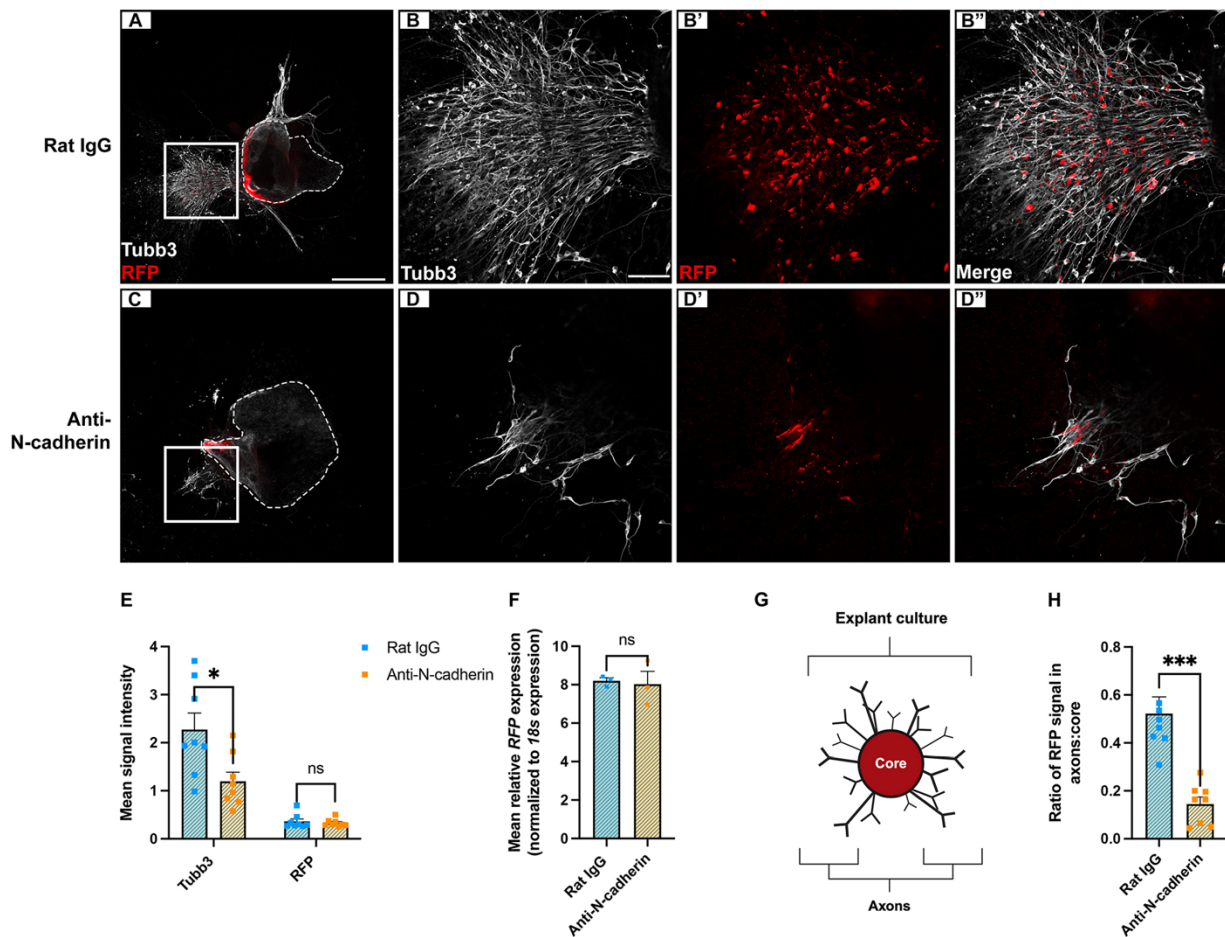


Fig. 3.11 The majority of placodal neurons rely on N-cadherin for axon outgrowth.

(A-D'') Representative images of trigeminal explant cultures with RFP-labeled placodal neurons after rat IgG (A-B'', n=8) or anti-N-cadherin (C-D'', n=8) treatment, and RFP and Tubb3 immunostaining. Areas outlined in A and C are shown at higher magnification in B-B'' and D-D'', respectively. Dotted lines in A and C indicate the perimeter of the explanted tissue. Scale bars: in A, 500 μ m for A and C; in B, 100 μ m for B-B'', D-D''. (E) Mean signal intensity for Tubb3 and RFP $\pm$ s.e.m. (F) Quantitative PCR for RFP normalized against chick 18s rRNA of rat IgG- and anti-N-cadherin-treated explants (n=3). (G) Trigeminal ganglion explant culture schematic, with cores (inside the explant) and axons (outside the explant) comprising the explant culture. Created in BioRender. McIntosh, A. (2025) <https://BioRender.com/gg9u4t2>. This figure was sublicensed

under [CC-BY 4.0](#) terms. (H) Ratio of RFP signal found within axons and cores $\pm$ s.e.m. Statistical significance was determined using unpaired t-tests and Mann–Whitney tests * $P<0.05$, *** $P<0.001$. Contributions from A. McIntosh.

3.4.7 Inhibiting N-cadherin-mediated adhesion diminishes neurite complexity in neural crest- derived neurons

Owing to the changes we observed in RFP-labeled placodal neurons after blocking N-cadherin-mediated adhesion, we next chose to assess whether neural crest-derived neurons were also impacted. To test this, a neural tube electroporation was performed with PiggyBacGFP with PBase to label the neural crest cells. Next, GFP-positive explant cultures were treated with either rat IgG or anti- N-cadherin as previously described (Fig. 3.7A) and immunostained with a GFP antibody, then neurites were traced (Fig. 12B-C'). Similar to our previous results after N-cadherin MO treatment (Fig. 3.8), we observed a decrease in neurite complexity after anti-N-cadherin treatment. Neural crest-derived neurons showed a significant reduction in both primary and secondary neurites compared to rat IgG-treated explants (Fig. 3.12D). In line with our results from Fig. 8, we did not observe a change in the average neurite length of primary neurites between treatment groups (Fig. 3.12E). Very few anti-N-cadherin-treated neural crest-derived neurons had secondary or tertiary neurites, preventing analyses of neurite length. These findings reveal that the adhesive role of N-cadherin impacts the ability of neural crest-derived neurons to generate complex neurites.

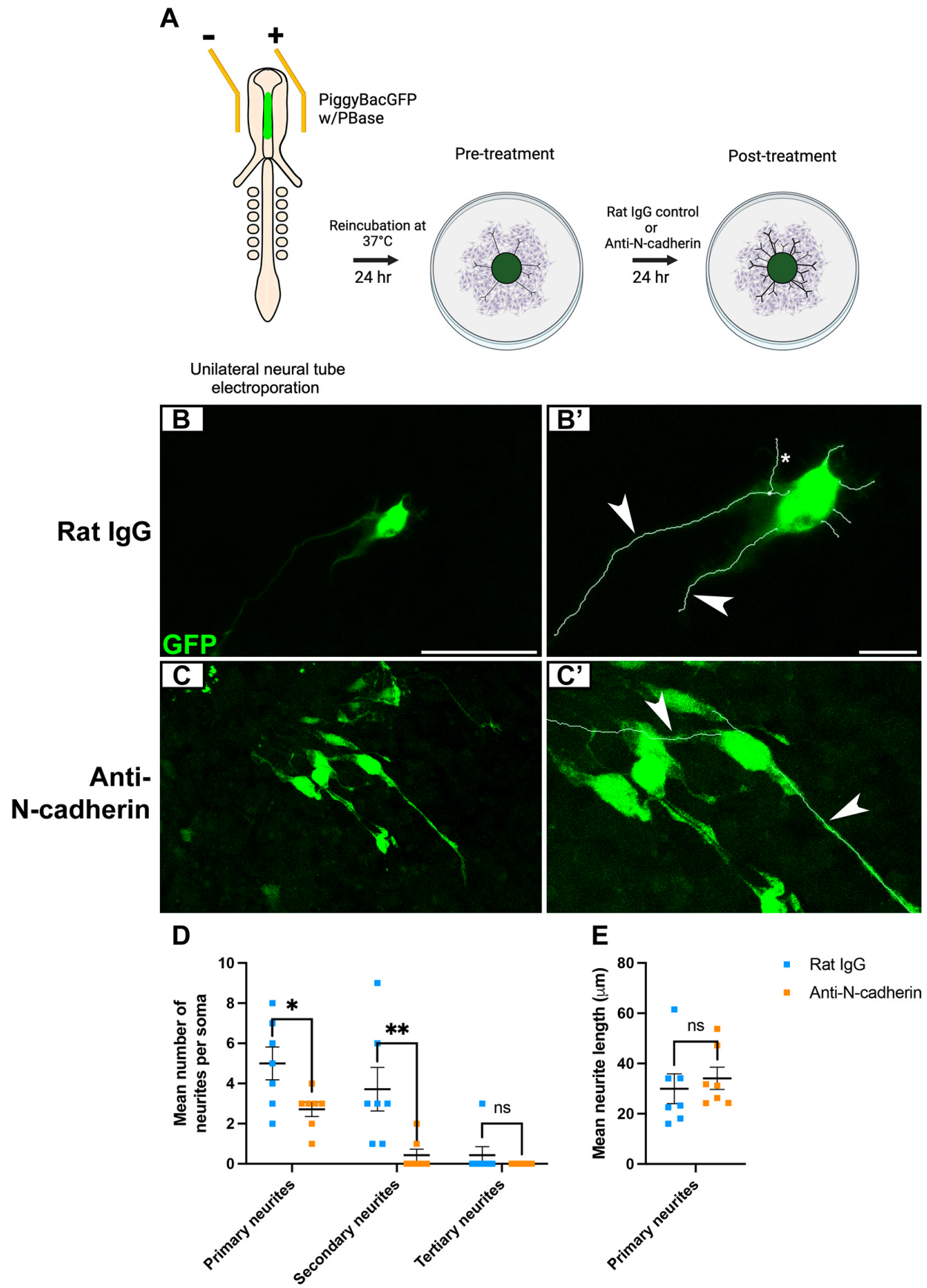


Fig. 3.12 Blocking N-cadherin-mediated adhesion reduces neurite complexity in neural crest-derived neurons.

(A) Schematic of N-cadherin function blocking assay after labeling neural crest-derived neurons with PiggyBacGFP. Created in BioRender. McIntosh, A. (2025) ([Gillig & Sanders, 2010](#); [Sanders, 2010](#); [Vermeiren et al., 2020](#)). This figure was sublicensed under [CC-BY 4.0](#) terms. (B,C) Representative images of trigeminal explant cultures with GFP-labeled neural crest-derived neurons after control rat IgG (B, n=7) or anti-N-cadherin (C, n=7) treatment. (B',C') GFP immunostaining and neurite traced images. Arrowheads and asterisks indicate examples of primary and secondary neurites, respectively. Scale bars: in B, 50 μ m for B,C; in B', 25 μ m for B',C'. (D,E) Number (D) and length (E) of GFP-positive neural crest-derived neurites after rat IgG or anti-N-cadherin treatment. Data are mean $\pm$ s.e.m. Statistical significance was determined using unpaired t-tests and Mann–Whitney tests. *P<0.05, **P<0.01; ns, not significant.

3.5 Discussion

Here, we demonstrate a sustained role for N-cadherin during trigeminal gangliogenesis, emphasizing the need for cell-cell adhesion in the formation and maturation of the OpV lobe of the trigeminal nerve. Our findings show that reducing N-cadherin from trigeminal placode cells and their neuronal derivatives disrupts trigeminal ganglion morphology and target innervation by specific OpV nerve branches. We found that neural crest-derived neurons rely on N-cadherin in placodal neurons for proper neurite outgrowth, and that β -catenin levels are reduced in them after N-cadherin knockdown in placode cells. Moreover, blocking N-cadherin-mediated adhesion led to similar effects, suggesting this underpins the *in vivo* phenotypes observed. These results highlight the continued role of N-cadherin for sensory neurodevelopment and reinforce the

importance of reciprocal interactions between placode cells and neural crest cells during cranial ganglia formation.

Depletion of N-cadherin from placodal neurons leads to persistent morphological defects in the developing trigeminal ganglion

N-cadherin in placode cells plays important roles during early cranial ganglia formation, including facilitating collective cell migration through a repulsive ‘chase and run’ mechanism (Theveneau et al., 2013) and the coalescence of undifferentiated neural crest cells and placodal neurons to generate the trigeminal ganglion (Shiau & Bronner-Fraser, 2009a). However, its function in regulating axon extension for proper target innervation has not been explored. Our experiments showed that N-cadherin knockdown initially increased trigeminal ganglion area, replicating previous results (Shiau & Bronner-Fraser, 2009a), but then led to a reduction in trigeminal ganglion size up to E4.5, which eventually recovered. We believe there are two potential reasons for this recovery. First, MOs are typically only stable for up to 4-5 days *in vivo*, reflecting a limitation of our technique (Bill et al., 2009). Therefore, it is possible that, at the later timepoints, the MO was targeted for degradation, minimizing phenotypic effects. However, we think this is unlikely, or plays a minimal role, as reduced N-cadherin protein levels were still observed at E6.5/HH28-30. Second, the continued differentiation of neural crest cells into neurons and glia beyond E4-5/HH24-27 (D’Amico-Martel & Noden, 1980) may compensate for this decrease in trigeminal ganglion size, which would not be discernable by Tubb3 immunostaining alone.

In keeping with previous studies, we observed more pronounced phenotypes in OpV compared to MmV, possibly due to the earlier differentiation of OpV neurons compared to MmV cells (Begbie, 2002; Graham & Shimeld, 2013; Lassiter et al., 2014b; McCabe et al., 2009; Stark et al., 1997b). Although this could be caused by the mosaic nature of the electroporation, we

suspect this difference is most likely attributed to MO dilution over time in MmV. OpV placode cells are the first to express neuron-specific markers and become post-mitotic, some even before leaving the ectoderm (McCabe et al., 2009), resulting in more prominent phenotypes. Conversely, MmV placode cells remain as proliferative neuroblasts until after delamination (Begbie, 2002; Graham & Shimeld, 2013; McCabe et al., 2009).

N-cadherin depletion from placode cells showed the greatest size difference in OpV at E3.5/HH19-22, coinciding with when the majority of placodal neurons have initiated axon outgrowth (Moody et al., 1989), while no change was observed by E6.5/HH28-30. This suggests that N-cadherin plays a role in promoting axon outgrowth during early stages of neurodevelopment. Notably, we did not observe changes to TrkA- and TrkB-expressing cells in OpV when these neurons are prominent (E5.5/HH26-27), indicating altered neuronal differentiation was not a factor in the morphological effects observed. Interestingly, subtle changes were sometimes noted in the subcellular distribution of these receptors after N-cadherin MO treatment. Further investigations would aid in understanding these phenomena. Moreover, N-cadherin protein levels were still reduced in OpV at E6.5/HH28-30, suggesting continuing effects on N-cadherin even if the MO was targeted for degradation. We also observed a decrease in cell division in OpV in this stage grouping after N-cadherin knockdown, pointing to other mechanisms that compensate for the absence of phenotype. As only a neuronal marker (Tubb3) was used in these experiments, it is possible that the balance of neural crest cell differentiation into neurons versus glia was affected, leading to more of the former than the latter.

Reducing N-cadherin in placodal neurons impacts OpV nerve innervation patterns

Further studies revealed a requirement for N-cadherin in the lateral and medial nasal branches of OpV, with the former exhibiting more drastic changes upon N-cadherin MO treatment.

The lateral nasal branch exhibited decreased terminal nerve branches, reduced nerve width and a smaller innervation area, while the medial nasal nerve showed similar trends for nerve width and innervation area, albeit to a lesser extent. Interestingly, there was no change in the number of terminal nerve branches in the medial nasal branch. This contrasting result – a smaller nerve bundle having similar axonal branching – suggests that N-cadherin functions to adhere individual axons with each other at medial nasal branch targets. Similar phenotypes after N-cadherin perturbations have also been observed in other systems (Reis et al., 2011; Shikanai et al., 2018; Tan et al., 2010).

Although both OpV branches begin developing at similar stages (Higashiyama & Kuratani, 2014), we chose to examine the E5.5/HH26-27 timepoint for the medial nasal nerve studies due to more consistency among embryos. While differences in embryo age could explain the varying results between the nerve branches, it is equally plausible that the proportion of N-cadherin-expressing placodal neurons differs between them, as the contribution of neural crest- and placode-derived neurons to these nerve branches is unknown. Further, the differences observed could be due to when the respective branches form, as our results suggest that medial nasal branch development occurs after the lateral nasal branch. If true, knockdown of N-cadherin would preferentially impact the lateral nasal nerve since it develops first. Alternatively, the mosaic nature of the electroporation technique may also explain these phenotypes.

N-cadherin in placodal neurons functions non-cell autonomously to control neurite production in neural crest-derived neurons

Given the observed alterations to trigeminal ganglion morphology, we investigated whether increased apoptosis contributed to these phenotypes. Although our Tubb3 immunohistochemistry results showed no size difference in OpV at the first timepoint examined (E2.5/HH16-18), a significant increase in apoptosis was detected at this timepoint. This suggests

that, while the same number of cells may initially contribute to the forming ganglion, some undergo apoptosis, potentially impacting OpV size at later stages and contributing to the long-term phenotypes seen in our studies. However, the absence of any increased apoptosis at later stages, particularly when neural crest cells are differentiating into neurons, implies other mechanisms are also involved.

To address this, we evaluated axon outgrowth of neural crest-derived neurons. Fluorescent labeling of neural crest cells followed by N-cadherin MO treatment in placode cells revealed very few neural crest-derived axons. The complexity of neurites in these neurons was significantly reduced, indicating non-cell autonomous effects. This highlights the continued importance of N-cadherin-mediated interactions throughout trigeminal gangliogenesis, particularly between placode- and neural crest-derived neurons. N-cadherin functions in neurite production for other peripheral neurons (Bixby & Zhang, 1990), and its localization at specific sites along the cell membrane precedes neurite formation (Gärtner et al., 2012). Depletion of N-cadherin from placodal neurons could prevent *trans* N-cadherin interactions between neural crest cells/ neural crest-derived neurons and placodal neurons, disrupting neurite production. Additionally, since N-cadherin is also involved in dendrite maintenance (Tan et al., 2010), the stability of neural crest-derived neurites may be compromised when N-cadherin is knocked down in placodal neurons. Interestingly, despite fewer neurites observed in N-cadherin MO-treated explants, neurite length remained the same between treatments, suggesting that processes controlling neurite length are unaffected by knockdown of N-cadherin in placode cells.

Irrespective of the mechanism behind reduced neurite formation, disruptions to the cytoskeleton are likely involved. Our studies revealed a decrease in β -catenin in neural crest-derived neurons upon knockdown of N-cadherin in placode cells. Membrane-bound N-cadherin

regulates the availability of both β -catenin and p120- catenin for binding and activating various signaling pathways (Radice, 2013). If *trans* N-cadherin interactions among neural crest and placode-derived neurons are disrupted, β -catenin could be impacted, leading to the loss of intracellular signaling required to activate GTPases and other cytoskeletal proteins, thus impacting neurite production. Finally, cadherin-receptor tyrosine kinases interactions at cellular membranes reportedly regulate each other (Chiasson-MacKenzie & McClatchey, 2018b), including those between N-cadherin and fibroblast growth factor receptors (Hansen et al., 2008; T. Nguyen & Mège, 2016a), which may be crucial for the proper growth, maturation and survival of neural crest-derived neurons. Disrupting N-cadherin levels could interfere with these interactions, affecting the proper development of these neurons.

N-cadherin-mediated adhesion facilitates axon outgrowth of placode- and neural crest-derived trigeminal neurons

To elucidate whether the *in vivo* phenotypes were due to loss of N-cadherin-dependent adhesion, we used a function-blocking antibody to broadly inhibit N-cadherin in trigeminal ganglion explant cultures. Three main conclusions emerged from the analysis. First, blocking N-cadherin-mediated adhesion did not preclude axon outgrowth. Second, intersection analyses revealed reduced axon outgrowth and branching in anti-N-cadherin-treated explants compared to rat IgG-treated controls. However, anti-N-cadherin-treated explants still extended axons to the periphery, though not significantly more than pre-treatment explants, suggesting some axons may be retracting, due to either pruning or lack of necessary factors in the media to support axon maintenance. Finally, segment lengths (used as a proxy for axons) were significantly reduced in our rat IgG-treated explants compared to both pre-treatment and anti-N-cadherin-treated explants. This reduction was due to a higher number of very short segments in the rat IgG group, as longer

axon lengths were observed in all groups, and argues against a substantial role for axon pruning. Since some trigeminal neurons could extend axons similarly to controls, despite N-cadherin inhibition, we conclude that N-cadherin is not essential for axon outgrowth in all trigeminal neurons. These data align with the *in vivo* observation for OpV branches in that N-cadherin knockdown does not completely block target innervation but reduces the number of axons reaching their targets. While this phenotype could be attributed to the mosaic nature of the MO electroporation, our *in vitro* results now offer another potential explanation, in that some neurons are not reliant on N-cadherin for the outgrowth of their axons.

We next identified the cellular origin of the neurons requiring N-cadherin-mediated adhesion for their outgrowth, hypothesizing that they would be placode derived since placode cell neuronal differentiation precedes neural crest cell differentiation *in vivo* (D'Amico-Martel & Noden, 1983). Our experiments showed reduced axon outgrowth in RFP-labeled placodal neurons after anti- N-cadherin-treatment. Although some neurons might have initially extended axons and then died, we believe this is unlikely because a subset of neurons was always capable of growing axons irrespective of when the anti-N-cadherin antibody was added to the explants. Although RFP transcript and protein levels were similar between treatments, very interestingly, RFP signal in axonal projections was reduced after anti-N-cadherin treatment, with most RFP signal originating from inside the cores. This indicates that N-cadherin promotes axon outgrowth, with its absence limiting the number of RFP-positive placodal neurons capable of extending axons.

Although we did not observe interactions between fluorescently labeled placodal neurons and neural crest-derived neurons *in vitro* upon blocking N-cadherin-mediated adhesion, we did see changes in neurite production of neural crest-derived neurons. While both primary and secondary neurite number was significantly reduced, neurite length remained unchanged across treatments.

These results mirrored phenotypes in neural crest-derived neurons when N-cadherin was knocked down from placode cells, indicating that the loss of N-cadherin from placodal neurons impacts the ability of neural crest-derived neurons to produce neurites. This likely contributes to the reduced size of the trigeminal ganglion observed in N-cadherin knockdown embryos, as these neurons may be unable to extend axons to their targets properly.

Surprisingly, a small subset of RFP-positive placodal neurons exhibited axon outgrowth after anti-N-cadherin treatment, potentially representing pioneer neurons of the trigeminal ganglion. Though pioneer neurons are well documented in invertebrate models (Clarke et al., 2020; Hidalgo & Brand, 1997; Klose & Bentley, 1989; D. M. Lin et al., 1995), evidence in vertebrates (Eisen et al., 1986; Ghosh et al., 1990; Gong & Shipley, 1995), including in mouse trigeminal neurodevelopment, suggests that the first axons to extend from OpV provide a path for secondary axons to reach their targets. A similar phenomenon has been observed in the chick trigeminal ganglion, where a few placode-derived neurons were observed to extend axons to the periphery, providing a path for later-born neurons to follow (Moody et al., 1989). Collectively, these studies and our new data suggest that a subset of placodal neurons may serve as pioneers upon which other placode-derived neurons and later neural crest-derived neurons ('followers') extend their axons (Moody et al., 1989). This N-cadherin-independent (pioneer) and -dependent (follower) outgrowth is reminiscent of mechanisms regulating migration in the facial branchiomotor neuron system (Wanner & Prince, 2013).

In this study, we have shown a sustained negative impact on the trigeminal ganglion following N-cadherin depletion from placodal neurons. Throughout development, including after neural crest cells differentiate into neurons, we observed changes in trigeminal ganglion size and reduced axon outgrowth to target tissues. These morphological differences could not be fully

explained by increased apoptosis. In support of this, neurite production and β -catenin levels in neural crest-derived neurons were altered after depleting N-cadherin from placode cells. Treatment of explants with an N-cadherin adhesion-blocking antibody caused similar effects on neurites, pointing to a non-cell autonomous requirement for N-cadherin in placodal neurons to facilitate axon outgrowth from placode- and neural crest-derived trigeminal neurons. Inhibiting N-cadherin-mediated adhesion *in vitro* also reduced axon outgrowth from most placodal neurons, demonstrating a cell-autonomous function for N-cadherin in this process. Altogether, our results add to the growing amount of evidence supporting the importance of reciprocal interactions between neural crest cells and placode cells during the formation of the cranial ganglia, and highlight a long-term, crucial role for N-cadherin during trigeminal gangliogenesis that will likely be translatable to other cranial ganglia and vertebrate species.

Chapter 4: Multiple glycoforms of TrkA interact with N-cadherin during trigeminal ganglion neurodevelopment

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4.1 Abstract

The trigeminal ganglion is a component of the sensory nervous system that arises from neural crest and placode cells. The dual origin of the trigeminal ganglion leads to a heterogeneous neuronal population that transmits somatosensory information from the face back to the brain. Proper trigeminal ganglion development relies, in part, on neurotrophic signaling, including interactions between Nerve Growth Factor and its cognate receptor, Tropomyosin receptor kinase A (TrkA), a receptor tyrosine kinase. Post-translational modifications, including glycosylation, play a crucial role in the ability of TrkA to reach the plasma membrane, yet the specific glycan profile and functional relevance of these modifications have not been characterized in sensory neurons *in vivo*. Here, we sought to characterize the different glycosylation events occurring on TrkA during trigeminal ganglion neurodevelopment. We discovered that multiple glycoforms of TrkA exist that correlate to partially and fully mature versions of the protein reported *in vitro*. Furthermore, we discovered that TrkA interacts with a cell adhesion molecule, N-cadherin, on membranes of trigeminal neurons, both in the cell bodies and axons. Based on the size of the TrkA bands that interact with N-cadherin, our results suggest these interactions are occurring both on the plasma membrane and intracellularly on the membrane of organelles. While interactions between receptor tyrosine kinases and cadherins have been shown in other contexts, our findings are the first to identify such an interaction in the trigeminal ganglion and suggest an important role

for coordination between neurotrophic signaling and cell adhesion for proper neurodevelopment, both during TrkA protein maturation and during receptor tyrosine kinase signaling. Given that aberrant receptor tyrosine kinase and cadherin signaling is commonly implicated in neurodevelopmental disorders and cancer, understanding how these interactions are established during normal development may provide additional insight into their dysregulation during disease.

4.2 Introduction

Cranial ganglia are components of the peripheral nervous system that transmit sensory information from the surrounding environment back to the central nervous system (Schlosser, 2018). The trigeminal ganglion, the largest of all cranial ganglia, extensively innervates the face to relay somatosensory information including pain, touch, and temperature (Heimer, 1983). It is derived from two embryonic cell populations, neural crest cells and placode cells, which originate in different anatomical locations but eventually become migratory and coalesce to form this sensory structure (D'Amico-Martel & Noden, 1980; Hamburger, 1961b). While both cell types differentiate into neurons, neural crest cells also give rise to supporting glia. This dual embryonic origin of trigeminal sensory neurons leads to a heterogeneous population with distinct molecular profiles and signaling responses (Bhuiyan et al., 2024; Marmigère & Ernfors, 2007; M. Q. Nguyen et al., 2017).

Among the signaling pathways that are critical for proper trigeminal ganglion development are those initiated downstream of neurotrophins and their cognate receptors, which regulate neuronal survival, outgrowth, and subtype specification (Barbacid, 1995; Cohen et al., 1954; Levi-Montalcini & Hamburger, 1953). As development progresses and trigeminal neurons respond to environmental cues, specific subclasses of neurons emerge including nociceptors,

mechanoreceptors, and thermoreceptors that detect pain, mechanical stimuli, and temperature, respectively. These neuronal subtypes can be identified in part by the expression of members of the receptor tyrosine kinase (RTK) family known as the tropomyosin receptor kinases (Trks), which are activated by secreted neurotrophic factors (Davies, 1997; Ernfors et al., 1992; Huang, Wilkinson, et al., 1999; Huang, Zang, et al., 1999; Mu et al., 1993).

TrkA, the high-affinity receptor for nerve growth factor (NGF), is expressed in the majority of nociceptive neurons (Fang et al., 2005). Upon ligand binding and subsequent receptor dimerization, TrkA undergoes autophosphorylation to activate downstream signaling pathways like MAPK, PI3K/Akt, and PLC γ , which promote neuronal survival and differentiation (Huang & Reichardt, 2003; Marlin & Li, 2015). Loss of NGF-TrkA signaling leads to increased apoptosis in small diameter neurons of both the trigeminal ganglion and dorsal root ganglion, confirming its essential role in neuronal survival and health during neurodevelopment (Johnson et al., 1980; Ruit et al., 1992; Smeyne et al., 1994). Although the function of TrkA has been characterized extensively in rat pheochromocytoma cells (PC12) (Negrini et al., 2013; Zhou et al., 1995) and models such as rodent (Fang et al., 2005; McMahon et al., 1994) and chick dorsal root ganglia (Gallo et al., 1997; George et al., 2010; Karlsson et al., 1998; Rifkin et al., 2000; R. Williams et al., 1995), far less is known about the post-translational mechanisms that regulate TrkA maturation, trafficking, and localization to the cell surface, which are critical for its function as a receptor. Existing information on these processes has been obtained exclusively from studies in PC12 cells, leaving these mechanisms unexplored *in vivo* within cranial sensory ganglia.

Proper localization of TrkA to the plasma membrane relies on a series of regulated processing and maturation steps (Jullien et al., 2002; Martin-Zanca et al., 1989; Watson et al., 1999; Zhou et al., 1995). Data from prior studies indicate that TrkA is synthesized as an immature,

unmodified precursor protein. In PC12 cells, the unglycosylated core TrkA protein is approximately 75 kD, although whether this remains true *in vivo* is unknown (Watson et al., 1999). Next, TrkA undergoes glycosylation in the ER to become a 110 kD precursor glycoprotein that undergoes further glycosylation in the Golgi to yield a 140 kD mature form prior to its transit to the cell membrane. Previous studies have shown that the 110 kD, partially glycosylated form of TrkA is N-linked glycosylated, while the 140 kD form contains additional sialic residues, likely on these N-linked glycans. Recently, a report has also suggested the presence of O-linked glycosylation on TrkA in cancer cells, though its presence and functional significance, particularly during normal development, are unclear (N.-Y. Lin et al., 2022).

In addition to RTKs, cell adhesion molecules such as cadherins are critical for neuronal organization and signaling. Cadherins regulate cell-cell adhesion in a calcium-dependent manner and contribute to tissue morphogenesis by influencing proliferation and intracellular signaling (Halbleib & Nelson, 2006b). Although multiple cadherins are noted in different tissues, neural cadherin (N-cadherin) is broadly expressed in the developing nervous system, including in trigeminal placode cells prior to their delamination, in their neuronal derivatives, and later in all trigeminal sensory neurons irrespective of cellular origin (Hatta & Takeichi, 1986b; Shiau et al., 2008a). Additionally, N-cadherin knockdown in trigeminal placode cells disrupts initial trigeminal ganglion condensation and later axon outgrowth through cell- and non-cell autonomous mechanisms involving adhesion (Halmi et al., 2025; Shiau & Bronner-Fraser, 2009a). Beyond their adhesive roles, cadherins can also interact with signaling receptors, including RTKs, to functionally regulate downstream signaling, receptor membrane localization, and cell behaviors such as migration and proliferation (Andl & Rustgi, 2005; Madarampalli et al., 2019a; T. Nguyen & Mège, 2016b; Qian et al., 2004a). Dysregulation of these RTK-cadherin interactions are

commonly seen in diseases such as cancer, where they contribute to the unchecked proliferation of cells. However, whether such RTK-cadherin interactions occur normally in the developing chick trigeminal ganglion, and how these interactions might influence trafficking and localization of receptors, has not yet been explored.

In this study, we investigate the glycosylation status of TrkA in chick trigeminal sensory neurons, including the relationship between TrkA and N-cadherin. Our results demonstrate that distinct TrkA glycoforms interact with N-cadherin in cell bodies versus axons, and these interactions occur on neuronal cellular membranes, likely on the plasma membrane and the membranes of organelles. Our findings reveal a previously uncharacterized interaction between neurotrophic signaling factor receptors and cell adhesion molecules that may regulate normal trafficking of TrkA to permit proper neuronal differentiation and survival during sensory ganglion development.

4.3 Materials and Methods

Chick embryos

Fertilized chicken eggs (*Gallus gallus*) were obtained from the Department of Animal and Avian Sciences at the University of Maryland (MD) and incubated at 37°C in humidified incubators on their sides. Embryos were incubated to reach desired Hamburger Hamilton (HH) stages based on times provided therein (Hamburger & Hamilton, 1992).

Immunohistochemistry

Embryos were collected at desired stages of interest and fixed in 4% paraformaldehyde (PFA) overnight at 4°C with agitation. After fixation, embryos were embedded and sectioned as described previously (Halmi et al., 2025). Briefly, embryos were processed through increasing

concentrations of sucrose (5% and 15%) and gelatin (7.5% and 20%), before being embedded in 20% gelatin. Embedded embryos were cryo-sectioned and immunostaining was performed (Halmi et al., 2025). Primary antibodies used were the following: anti-TrkA (a gift from Dr Francis Lefcort, Montana State University, Bozeman, MT, USA; 1:5000) and anti-N-cadherin (6B3, DSHB, 1:75). Secondary antibodies (goat anti-rabbit IgG and goat anti-mouse IgG₁) were used at 1:250.

Explant immunocytochemistry

Explant slides were washed once with 1X PBS, followed by fixation in 4% PFA for 30 minutes at room temperature with agitation. After fixation, slides were rinsed with 1X PBS and stored in 1X PBS at 4°C until ready for immunocytochemistry. A hydrophobic perimeter was drawn around the slides using an ImmEdge Pen (Vector Laboratories) to prevent dehydration of cells, and explants were taken through blocking, primary antibody incubation, and secondary antibody incubation in the absence of coverslips, as described in the immunohistochemistry methods. Slides were mounted with DAPI-fluoromount media (SouthernBiotech) and covered with a glass coverslip.

Confocal imaging

All images were acquired with the LSM Zeiss 800 confocal microscope. Laser power, gain and offset were kept consistent for the different channels during all experiments where possible.

Adobe Photoshop CC 2025 was used for image processing.

Immunoprecipitation

Wild-type E6.5/HH28-30 trigeminal ganglia were dissected from embryos and cell bodies were separated from axons using fine tungsten needles. Samples were centrifuged for 5 minutes at 4°C at 2292 xg. Pellets were flash frozen in liquid nitrogen and stored at -80°C. When needed, samples were lysed in lysis buffer (50 mM Tris (pH 7.5), 100 mM NaCl and 0.5% IGEPAL CA-630))

supplemented with cOmplete Protease Inhibitor Cocktail (Roche) and 1 mM PMSF for 30 minutes on ice with periodic gentle vortexing. Lysates were then centrifuged at 20,627 xg for 20 minutes at 4°C. The soluble protein fraction was then quantified by Bradford assay (ThermoFisher). N-cadherin-TrkA coimmunoprecipitations were performed with Protein A/G magnetic beads (ThermoFisher) according to the manufacturer's instructions. Briefly, equivalent amounts of protein lysates were incubated overnight with 10 µg of either the N-cadherin antibody (Abcam, ab182) or a rabbit IgG antibody (R&D Systems, AB-105-C) at 4°C with constant mixing. The following day, the lysates were combined with washed magnetic beads (3 times in wash buffer) and incubated for 1 hour at room temperature with constant mixing. After this, the bound proteins were washed on the beads three times before being eluted off by boiling at 99°C for 7 minutes in 1X reducing Laemmli sample buffer.

Trigeminal ganglion explant cultures

Wild-type embryonic day (E)6.5/HH28-30 embryos were removed from eggs and stored on ice in neuronal dissection media (1X Hank's Balanced Salt solution with 1% pen/strep, 10 mM HEPES, and 1.4 mM glucose). Trigeminal ganglia were dissected on a sylgard-coated dish by first bisecting the embryo and then removing the trigeminal ganglion and axonal projections with tungsten needles. Dissected ganglia were dispersed with a P10 pipette and cultured on two-well glass chamber slides (ThermoFisher) coated with Poly-L-Lysine (0.01% PLL, Sigma) and fibronectin (0.01%, Sigma). To make the coated slides, PLL was added to each chamber on the glass slides and incubated inside a biosafety cabinet for 30 minutes at room temperature. The PLL was removed, and slides were washed three times with water. Next, fibronectin diluted in DMEM media (ThermoFisher) was added to each chamber and incubated at 37°C for a minimum of 2 hours. The fibronectin solution was removed, and slides were stored at 4°C until needed (for up to

1 week). Explants were grown in Neurobasal complete media (ThermoFisher) supplemented with B27 (1%, ThermoFisher), N2 (0.5%, ThermoFisher), Glutamax (1%, ThermoFisher), penicillin/streptomycin (1%, ThermoFisher), and with nerve growth factor (ThermoFisher), brain-derived neurotrophic factor (Amgen) and neurotrophin-3 (Amgen), all at 50 ng/ml.

Immunoblotting

Samples were separated by 8% SDS-PAGE, then transferred to a nitrocellulose membrane (BioRad). The membrane was blocked in 5% milk (in 1X PBS+0.1% Tween-20 (PTW)) for 30 minutes at room temperature followed by overnight incubation at 4°C with the following primary antibodies diluted in blocking solution: N-cadherin (MNCD2, DSHB 1:100) and TrkA (MSU, 1:5000 or Origene, 1:1000). Membranes were washed three times for 10 minutes each in PTW before incubation with secondary antibodies (species- and isotype-specific horseradish peroxidase, Jackson ImmunoResearch) diluted in blocking solution for 1 hour at room temperature. Membranes were washed three times for 10 minutes each in PTW. Antibody detection was performed using Supersignal West Pico or Femto chemiluminescent substrates (ThermoFisher) and visualized using a ChemiDoc XRS system (Bio-Rad). To calculate protein molecular weights, a straight vertical line was drawn through the protein ladder and a profile plot was generated on Fiji (Schindelin et al., 2012). The pixel value for each peak, which corresponded to a molecular weight marker was recorded. The $\log_{(10)}$ value for each molecular weight marker was calculated and a graph was generated with the migration distance (pixel value) along the x-axis and the $\log_{(10)}$ value along the y-axis. A linear regression was fitted and the corresponding equation of the line was used to calculate the molecular weight of all unknown bands.

Enzymatic treatments of lysate

For all enzymatic treatments, equal amounts of trigeminal ganglia lysate were used. PNGase F (NEB, P0704S), O-glycosidase (NEB, P0733S), and α 2-3,6,8 Neuraminidase (NEB, P0720S) were added to lysate per manufacturer's guidelines. Samples were incubated for 2-4 hours at 37°C. After incubation, 1X reducing Laemmli sample buffer was added to each sample, and samples were boiled at 99°C for 7 minutes before protein separation by SDS-PAGE.

Glycoprotein enrichment

Concanavalin A (ConA) and Wheat Germ Agglutinin (WGA): To enrich for N-linked glycans and sialic acid residues, ConA and WGA Glycoprotein Isolation kits were used, respectively (ThermoFisher, 89804, 89805). Briefly, the resin was first washed three times with 1X Wash Buffer. Trigeminal ganglia lysate was added to the washed resin and 1X Wash Buffer was added to reach a final volume of 250 μ L. Lysate/resin mixtures were incubated for 1 hour at room temperature with constant mixing. After incubation, glycoprotein-bound resin was washed four times with 1X Wash Buffer. After the final wash, 1X reducing Laemmli sample buffer was added to each sample, and samples were boiled at 99°C for 7 minutes before protein separation by SDS-PAGE. This experiment was performed on unconjugated agarose resin as described above for a control.

Peanut Agglutinin (PNA): To enrich for O-linked glycans, biotinylated PNA (Vector Laboratories, B-1075-5) was used. Prior to enrichment, the lysate was treated with α 2-3,6,8 Neuraminidase (NEB, P0720S) to remove sialic acid residues. In a microcentrifuge tube, neuraminidase-treated lysate, biotinylated PNA (10 μ g/mL), and Wash/Binding Buffer (1X PBS with 0.05% Tween-20) were combined to reach a volume of 500 μ L and incubated overnight at 4°C with constant mixing. The next day, streptavidin Sepharose resin (ThermoFisher, 20349) was washed with Wash/Binding Buffer three times before adding the lysate/lectin solution to it.

Lysate/resin mixtures were incubated for 1 hour at room temperature with constant mixing. After incubation, glycoprotein-bound resin was washed four times with Wash/Binding Buffer. After the final wash, 1X reducing Laemmli sample buffer was added to the sample, which was then boiled at 99°C for 7 minutes before protein separation by SDS-PAGE. This experiment was performed on Sepharose resin without biotinylated PNA as described above for a control.

Inhibitor treatment of explant cultures

Wild-type E6.5/HH28-30 trigeminal ganglia explant cultures were grown for 1 day *in vitro* as described in the explant cultures section. Tunicamycin (Sigma, T7765), Brefeldin A (Sigma, B6542), and Monensin (Sigma, M5273) were resuspended in DMSO to obtain a concentration of 1 mg/mL. The next day, all inhibitors, and the DMSO control, were separately added to Neurobasal complete media with brain-derived neurotrophic factor (Amgen, 50 ng/ml) and neurotrophin-3 (Amgen, 50 ng/ml), at a final concentration of 1 µg/mL, and each inhibitor (or control) was added to the explant cultures for 4 hours. After incubation, the media was removed, and explants were washed in 1X PBS before either being fixed in 4% PFA for immunostaining or flash frozen in liquid nitrogen and stored at -80°C to be processed for immunoblotting.

Extraction of membrane and cytosolic protein fractions from trigeminal ganglia

Membrane and cytosolic proteins from E6.5/HH28-30 trigeminal ganglia were isolated using the Mem-PER Plus Membrane Protein Extraction Kit (ThermoFisher 89842), per manufacturer's guidelines. Briefly, flash-frozen trigeminal ganglia were thawed on ice and washed once with Cell Wash Solution. Permeabilization Buffer was added to trigeminal ganglia, mixed until a homogenous solution was obtained, and incubated for 10 minutes at 4°C with constant mixing. The solution was centrifuged at 16,000 xg for 15 minutes at 4°C to pellet permeabilized cells. The supernatant which contained the cytosolic protein fraction was removed and transferred to a new

tube. Solubilization Buffer was then added to the pellet containing the membrane-bound proteins and mixed until a homogenous solution was obtained. The solution was incubated for 30 minutes at 4°C with constant mixing before being centrifuged at 16,000 xg for 15 minutes at 4°C. The supernatant containing membrane-associated proteins was transferred to a new tube before both fractions were quantified by Bradford assay for subsequent experimentation.

4.4 Results

4.4.1 TrkA possesses multiple glycoforms in the trigeminal ganglion

Previous work has demonstrated the glycosylation of TrkA in PC12 cells, which serve as an *in vitro* model of neuronal differentiation. However, TrkA glycosylation has not been studied *in vivo*. Thus, we first sought to establish what types of glycans were present on TrkA in chick trigeminal neurons. We treated trigeminal ganglia lysate with enzymes that remove specific sugar modifications on proteins (PNGase F: N-linked glycans, O-glycosidase: Core 1 and Core 3 O-linked glycans, and neuraminidase: sialic acids) and assessed changes to TrkA banding patterns by immunoblotting (Fig. 4.1A). In both the input and mock control lanes, the latter containing the enzymatic buffers added to the lysate without any enzymes, we observed six bands: the common doublet observed for TrkA, which we calculated to be 137 and 107 kD, as well as multiple lower molecular weight bands that were 93 kD, 76 kD, 59 kD, and 53 kD. Notably, in some experiments, we also observed a 223 kD TrkA band in the input that was reduced in molecular weight in the mock control treatment (Fig. 4.2).

Upon PNGase F treatment, we observed a total collapse of the 137 kD, 107 kD, and 93 kD bands to a singular 88 kD band, as well as two lower molecular weight bands at 67 kD and 56 kD, and the persistence of the 53 kD band. After neuraminidase treatment, we observed a slight shift

in the 137 kD band down to 127 kD, and a slight reduction in size of the 93 kD band to 89 kD, but no change in the 107 kD, 76 kD, 59 kD, or 53 kD bands. Since sialic acid residues are often found at the end of O-glycans and mask the ability of O-glycosidase to cleave these glycans completely, O-glycosidase and neuraminidase treatment were performed together. Interestingly, we observed a prominent 177 kD band, in addition to the other band shifts observed in the neuraminidase only-treated lysate. When PNGase F and neuraminidase were used together, we observed the same results as the PNGase F only treatment. Lastly, in the combination treatment with PNGase F, O-glycosidase, and neuraminidase, we observed the 177 kD band we had seen previously after O-glycosidase + neuraminidase treatment, a prominent band at 88 kD, which we observed after PNGase F treatment, and two lower bands that were 63 kD and 52 kD in size (Fig. 4.1A). We also examined N-cadherin, whose banding patterns only changed in the presence of PNGase F (Fig. 4.3). This is in line with other reports indicating that N-cadherin is only modified with N-linked glycans (Guo et al., 2009; Langer et al., 2012). Altogether, these enzyme treatments confirm that chick TrkA possesses multiple different types of sugars, including N-linked and sialic acid modifications, and indicate that the 107 kD TrkA band contains N-linked glycans while the 137 kD band is both N-linked and sialylated. Our results also suggest that O-linked glycosylation might be evident due to the presence of a 177 kD band after O-glycosidase + neuraminidase treatment.

To further explore the glycosylation profile of TrkA, we next performed experiments to enrich for different types of glycosylations through the use of Concanavalin A (ConA), which binds alpha-linked mannose or terminal glucose residues that are associated with N-linked glycosylation; Wheat Germ Agglutinin (WGA), which recognizes N-acetyl-glucosamine groups or sialic acid; and Peanut Agglutinin (PNA), which binds galactosyl-(β -1,3)-N-acetyl-galactosamine, a component of the O-glycosylation pathway. Interestingly, TrkA was enriched after all three

treatments, further supporting the presence of O-linked glycans on TrkA (Fig. 4.1B). In addition to the 137 kD, 107 kD, 93 kD, and 76 kD TrkA bands observed in Fig. 4.1A, ConA and WGA bound glycosylations on two higher molecular weight TrkA bands at 223 kD and 177 kD. PNA also pulled down the 223 kD band but did not enrich for as many bands as the ConA or WGA treatment. Instead, PNA bound a 117 kD form of TrkA, along with the 107 kD TrkA glycoform seen in other treatments. These experiments confirm that TrkA contains N-linked glycans and sialic acid residues, in line with data from PC12 cells, but also reveal, for the first time, additional O-linked glycosylation in chick sensory neurons. These results suggest that while similar post-translational modifications occur on TrkA *in vitro* and *in vivo*, additional modifications are also present in chick trigeminal ganglion neurons.

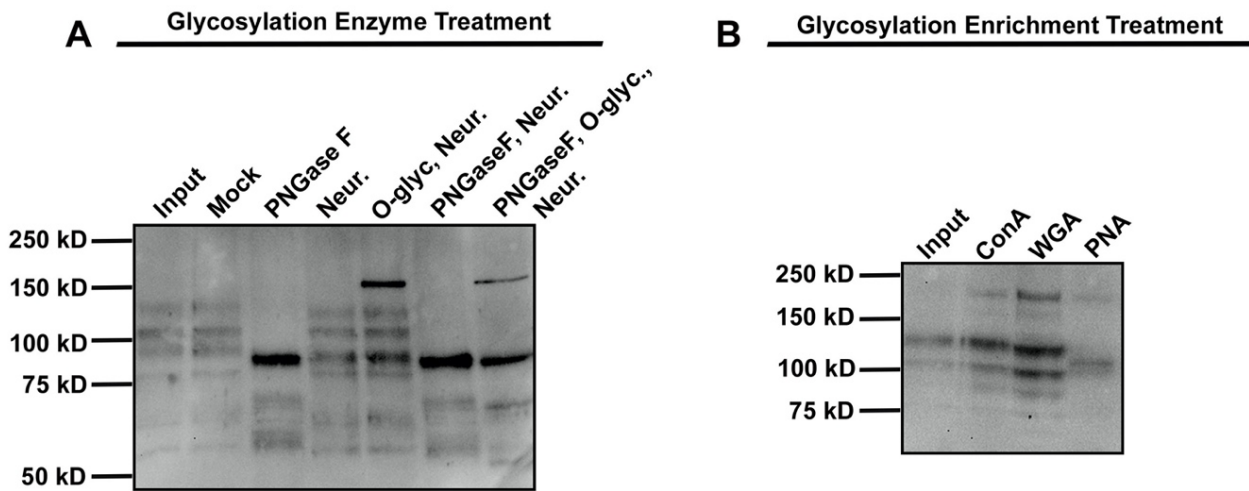


Fig. 4.1 Different post-translational modifications occur on TrkA.

(A) E6.5/HH28-30 trigeminal ganglia lysate treated with enzymes to inhibit glycosylation followed by immunoblotting for TrkA (N=3). Input: Untreated trigeminal ganglia lysate. Mock (control): Lysate mixed with enzyme buffers. PNGase F, Neuraminidase, O-glycosidase (O-glyc.).

(B) E6.5/HH28-30 trigeminal ganglia lysate after glycan enrichment followed by immunoblotting

for TrkA (N=3). Input: Untreated trigeminal ganglia lysate. Concanavalin A (ConA), Wheat Germ Agglutinin (WGA), Peanut Agglutinin (PNA).

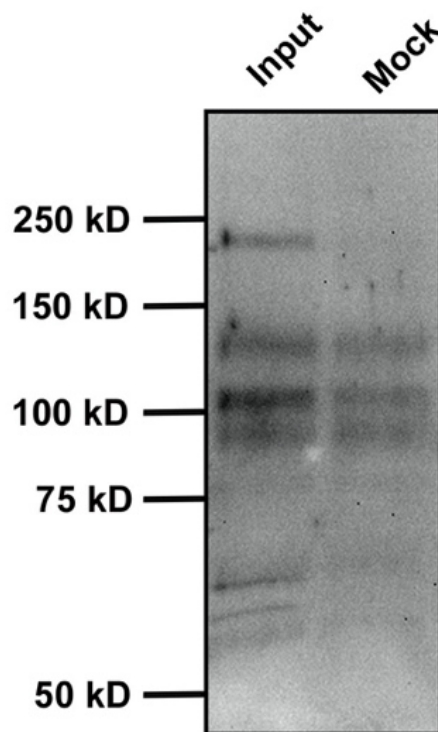


Fig. 4.2 Higher molecular weight TrkA is observed in trigeminal ganglia lysate.

E6.5/HH28-30 trigeminal ganglia lysate treated with enzymes to inhibit glycosylation followed by immunoblotting for TrkA. Input: Untreated trigeminal ganglia lysate. Mock (control): Lysate mixed with enzyme buffers (N=3).

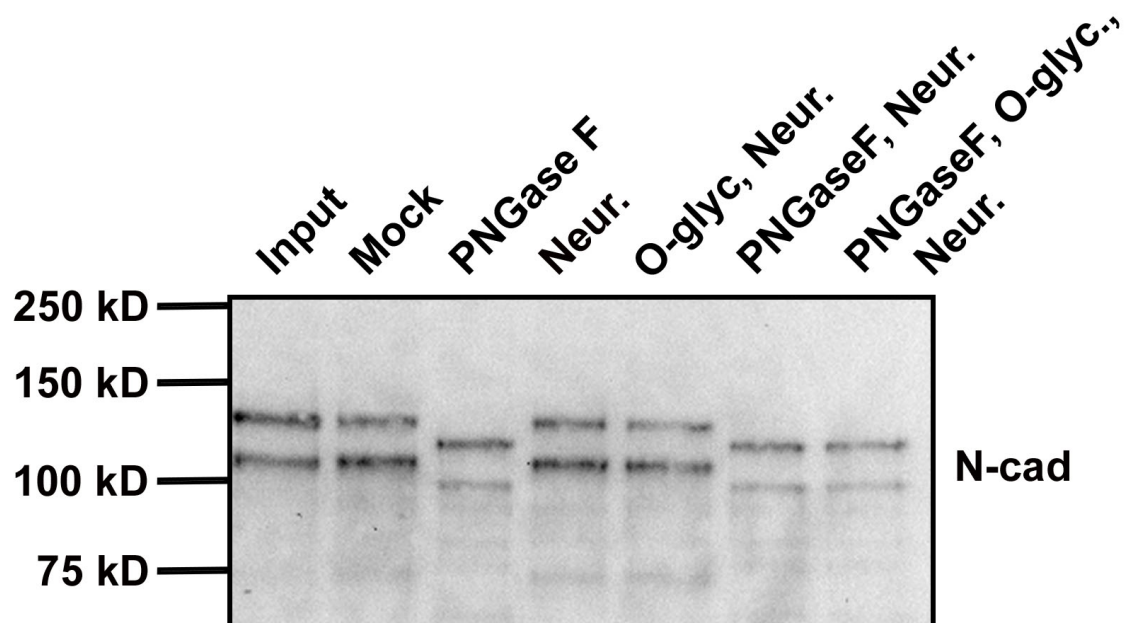


Fig. 4.3 N-cadherin is N-linked glycosylated.

E6.5/HH28-30 trigeminal ganglia lysate treated with enzymes to inhibit glycosylation followed by immunoblotting for N-cadherin (N-cad). Input: Untreated trigeminal ganglia lysate. Mock (control): Lysate mixed with enzyme buffers. PNGase F, Neuraminidase, O-glycosidase (O-glyc.) (N=2).

4.4.2 Inhibiting components of the secretory pathway impacts TrkA processing

To further explore the role of glycan processing of TrkA, we treated trigeminal neuronal explant cultures with validated inhibitors of protein processing and assessed TrkA localization and expression via immunostaining and immunoblotting. We hypothesized that each inhibitor would yield a unique change in TrkA expression and/or localization depending upon which portion of the secretory pathway was perturbed. Although we observed multiple TrkA bands, we chose to focus only on the partially and fully glycosylated forms for our analyses, as these are the most prevalent forms that move through the secretory pathway. In the DMSO control-treated explants, we observed TrkA perinuclearly as well as along the cell membrane (Fig. 4.4A-A'), and the typical doublet (107 kD and 137 kD) observed for TrkA that correspond to the partially and fully glycosylated forms (Fig. 4.4E). We also observed the 223 kD TrkA form in all of our conditions. Interestingly, in these experiments, TrkA ran slightly differently, with the partially and fully glycosylated forms appearing at 98 kD and 122 kD, respectively. Monensin is an antibiotic that inhibits the export of proteins out of the Golgi (Griffiths et al., 1983; Hammerschlag et al., 1982). This treatment yielded an accumulation of TrkA in a perinuclear location, possibly the Golgi given its subcellular distribution and the mechanism of action of Monensin (Fig. 4.4B-B'). While we observed the typical banding pattern for TrkA by immunoblotting, both the 98 kD and 122 kD forms were reduced, with the fully glycosylated form of TrkA being more greatly impacted than the partially glycosylated form (6.5-fold decrease vs. 1.5-fold decrease, Fig. 4.4E-G). Brefeldin A is a fungal metabolite that inhibits the anterograde transport of proteins from the ER to the Golgi, causing proteins to accumulate in the ER (Chardin & McCormick, 1999). After Brefeldin A treatment, we observed diffuse expression of TrkA throughout the cytoplasm of neuronal cell bodies (Fig. 4.4C-C'). This result correlated with the presence of a very prominent 98 kD TrkA

band after immunoblotting (7-fold increase) and a reduction in the intensity of the 122 kD band (10-fold decrease, Fig. 4.4E-G). Lastly, Tunicamycin is an antibiotic that inhibits N-linked glycosylation of proteins in the ER, leading to an accumulation of unfolded proteins and cellular stress (Struck et al., 1978; Takatsuki et al., 1971). After Tunicamycin treatment, we observed a reduction in overall expression of TrkA in the neuronal cultures and their axonal protrusions (Fig. 4.4D-D'). This phenotype is in line with what we observed via immunoblotting, in that overall TrkA levels were decreased (2-fold decrease in the 122 kD band, 5-fold decrease in the 98 kD band, Fig. 4.4E-G). Taken together, these experiments indicate that blocking the secretory pathway at distinct points alters TrkA distribution and expression, correlating with the portion of the pathway that was perturbed.

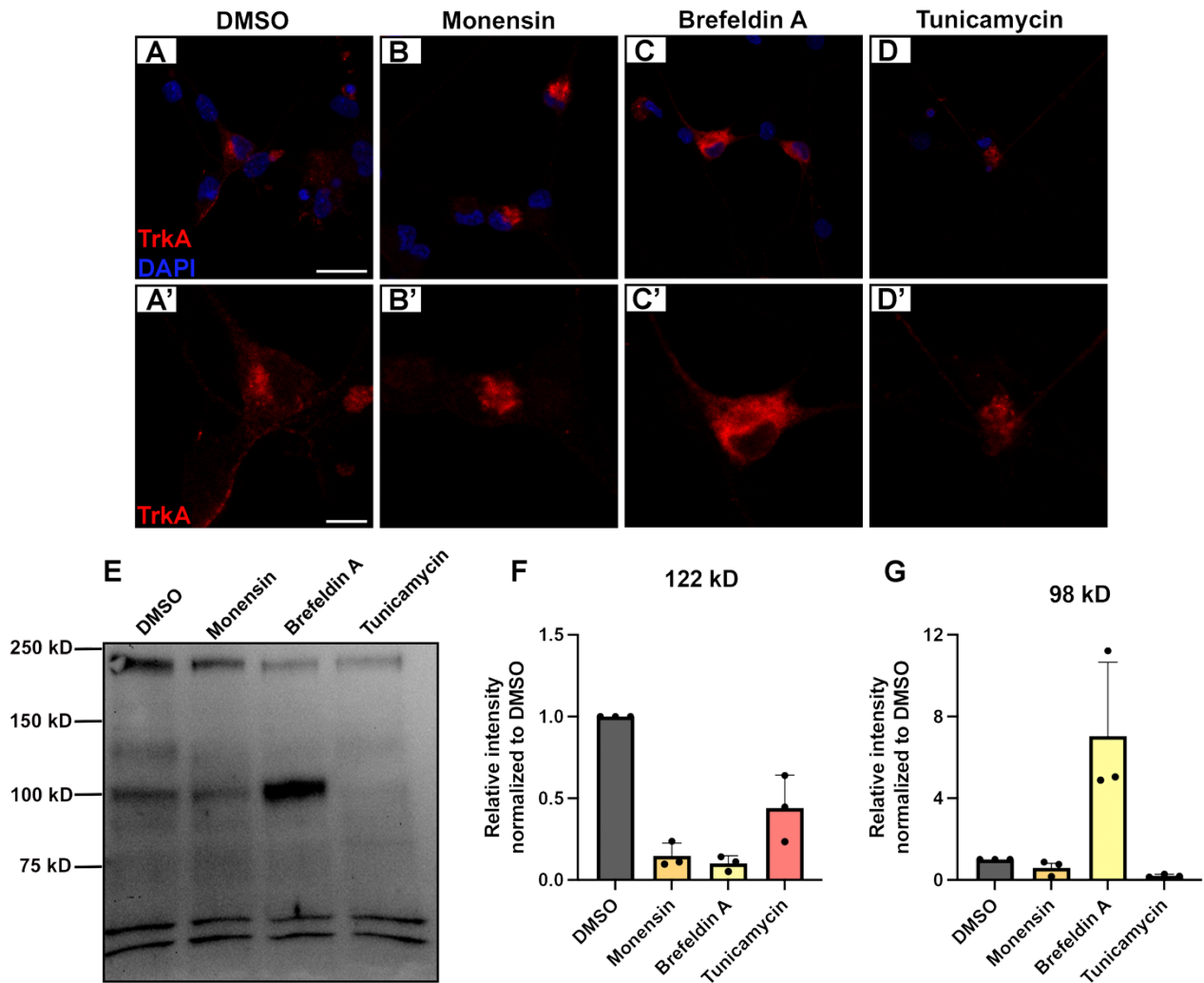


Fig. 4.4 Inhibitors of the secretory pathway disrupt TrkA expression and glycosylation within trigeminal neurons.

(A-D') Representative images of cultured trigeminal ganglia following treatment with DMSO (A-A'), Monensin (B-B'), Brefeldin A (C-C'), or Tunicamycin (D-D'), and immunocytochemistry for TrkA and DAPI (marks nuclei). Scale bar in (A) is 25 μ m and applies to (B, C, D). Scale bar in (A') is 10 μ m and applies to (B', C', D'). (E) Immunoblot for TrkA using lysate prepared from trigeminal ganglia explant cultures following treatment with DMSO, Monensin, Brefeldin A, and Tunicamycin. (F-G) Quantification of 122 kD (F) and 98 kD (G) TrkA bands normalized against DMSO control represented as averages $\pm$ SD (N=3).

4.4.3 N-cadherin and TrkA show distinct expression in trigeminal neurons

To investigate possible interactions between N-cadherin and TrkA in chick trigeminal sensory neurons, we next examined the expression pattern of TrkA, and whether it colocalized with N-cadherin, both in the embryo and in trigeminal ganglion explant cultures. Immunostaining was used to evaluate spatial distribution within trigeminal neurons, and immunoblotting was conducted to assess protein levels and sizes. In the embryo, all trigeminal neurons express N-cadherin (Fig. 4.5A, A'), while only a subset express TrkA (Fig. 4.5B, B'), as expected (Halmi et al., 2025; Leonard et al., 2022). We noticed colocalization of the two proteins in certain areas within trigeminal neurons *in vivo*, including at the cell membrane and in axons (Fig. 4.5C, C', arrowheads). In trigeminal ganglia explant cultures, we saw that the majority of N-cadherin staining was found at the membrane (Fig. 4.5D, G), while the majority of TrkA staining was cytoplasmic (Fig. 4.5E, H). However, we also observed certain areas within these explants where N-cadherin and TrkA appeared to colocalize at the plasma membrane and intracellularly in apparent perinuclear vesicles (Fig. 4.5F, I, arrowheads).

When examining TrkA by immunoblotting, we observed similar banding patterns and molecular weights *in vivo* (Fig. 4.5J) and *ex vivo* (Fig. 4.3K) - 76 kD, 93 kD, 107 kD, and 137 kD - as observed previously (Fig. 4.1). We also noted two higher molecular weight bands at 223 kD and 177 kD in our *ex vivo* lysate, as seen in our glycan enrichment experiments (Fig. 4.1B). Collectively, these findings indicate that TrkA colocalizes with N-cadherin in trigeminal neurons and maintains similar, but not identical, banding patterns both *in vivo* and *ex vivo*.

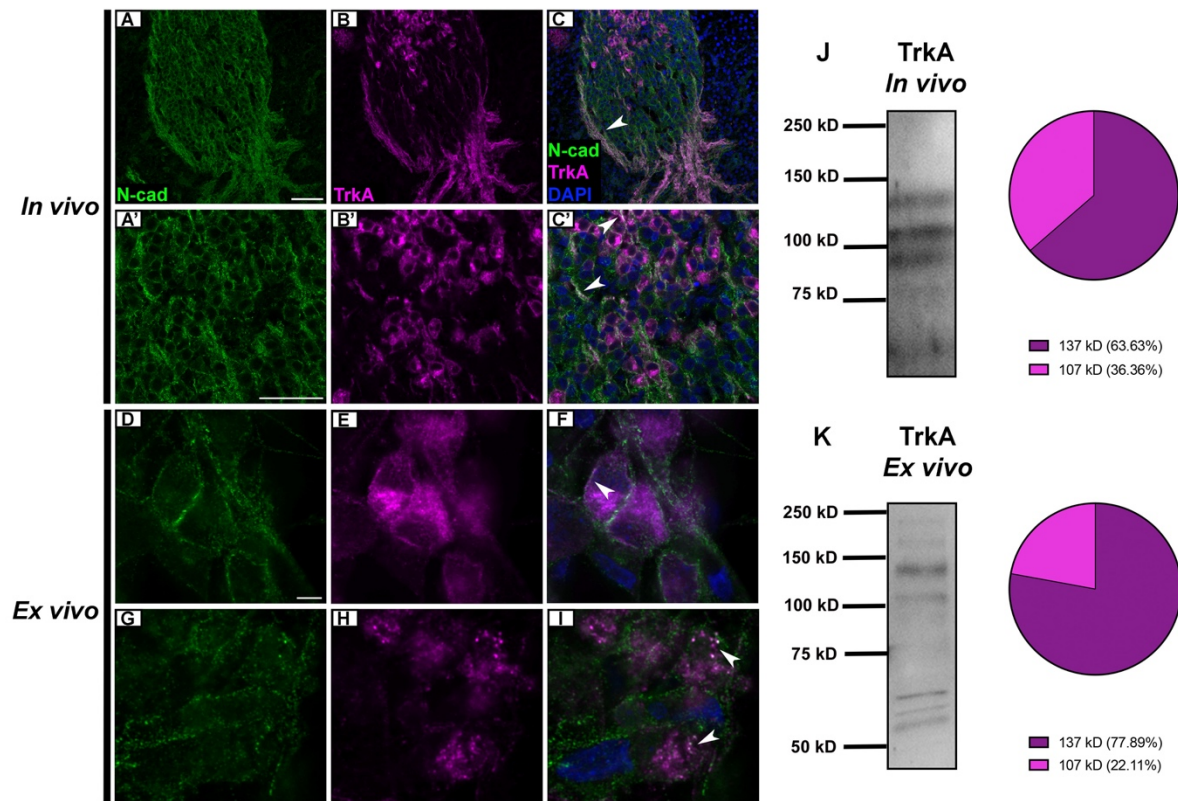


Fig. 4.5 N-cadherin and TrkA are coexpressed in trigeminal neurons.

(A-C') Representative transverse sections through the trigeminal ganglion of an E5.5/HH28 chick embryo following immunohistochemistry for N-cadherin (N-cad) and TrkA. Scale bar in (A) is 50 μ m and applies to (B, C). Scale bar in (A') is 50 μ m and applies to (B', C'). (D-I) Representative trigeminal ganglia explant cultures after immunocytochemistry for N-cadherin and TrkA. Scale bar in (D) is 25 μ m and applies to (E-I). Arrowheads indicate colocalization of N-cadherin and TrkA. (J, K) Immunoblots of HH28-30 trigeminal ganglia for TrkA with lysate from the embryo (*in vivo*) (J) and from trigeminal ganglia explant cultures (*ex vivo*) (K). Pie charts represent percentage of total signal coming from the different bands of TrkA.

4.4.4 N-cadherin biochemically interacts with TrkA in trigeminal neurons

Due to the colocalization of N-cadherin and TrkA that we observed by immunostaining, we sought to determine whether these two proteins physically interact in the chick trigeminal ganglion. To test this, we used a co-immunoprecipitation assay and examined neuronal cell bodies separately from their axonal projections, as we hypothesized that different forms of TrkA would be expressed in neuronal cell bodies where protein processing occurs versus axons where the mature receptor functions in signaling with NGF. Trigeminal ganglia and their nerve branches were dissected from chick embryos, and once removed, a crude cut was made at the base of the OpV and MmV lobes to separate trigeminal cell bodies from axons (Fig. 4.6A, dotted lines). Once lysates were generated for trigeminal cell bodies and axons, an N-cadherin immunoprecipitation with a validated N-cadherin antibody (Halmi et al., 2022) was performed followed by TrkA immunoblotting to detect possible interactions. In the cell bodies experiment, we observed the 223 kD, 137 kD, and 107 kD TrkA bands in the input, all of which interact with N-cadherin (Fig. 4.6B). This differed subtly from what we noted in the axons, where only the 223 kD and 137 kD bands were present in the input and after N-cadherin immunoprecipitation (Fig. 4.6C). Interestingly, in both immunoprecipitations, we also observed two smaller TrkA bands at 75 kD and 58 kD. These results align with our hypothesis and indicate that N-cadherin interacts with different forms of TrkA within different compartments of trigeminal neurons.

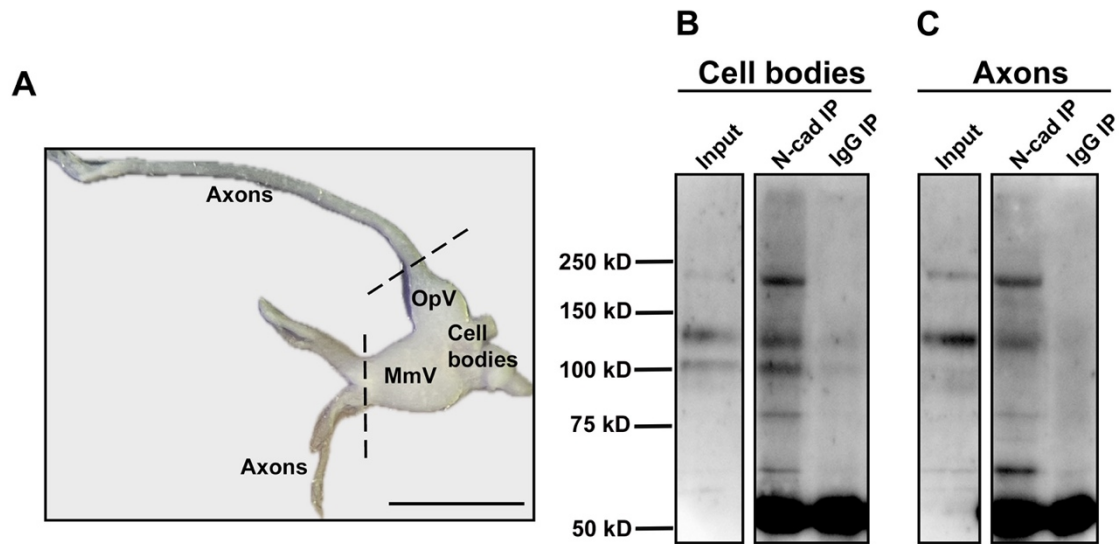


Fig. 4.6 Different glycosylated forms of TrkA physically interact with N-cadherin in distinct compartments of the trigeminal ganglion.

(A) Dissected trigeminal ganglion from an E5.5/HH28 chick embryo. Dotted lines indicate approximate location where cuts were made to separate trigeminal cell bodies from axons. Scale bar is 1mm. (B-C) N-cadherin-TrkA co-immunoprecipitation on pooled HH28-30 trigeminal cell bodies and axons. Input lanes contain 10% of lysate loaded in immunoprecipitation lanes. OpV, ophthalmic; MmV, maxillomandibular; IP, immunoprecipitation; IgG, Immunoglobulin G. N=3.

4.4.5 TrkA and N-cadherin interact on the membranes of trigeminal neurons

Our results demonstrate that N-cadherin interacts with the 107 kD N-linked glycosylated, 137 kD N-linked and sialylated, and 223 kD glycoforms of TrkA. However, because only the fully glycosylated form of TrkA is thought to function at the neuronal plasma membrane in signaling with NGF, these results suggest that the interactions we observed between N-cadherin and TrkA are occurring at least partially intracellularly, and potentially in the organelles in which these modifications take place. To further explore this, we separated membrane-bound and membrane-associated proteins from cytosolic proteins in E6.5/HH28-30 trigeminal ganglia. We first verified the accuracy of our extraction method by probing for different proteins and examining in which fraction they were observed. Both N-cadherin and TrkA appeared solely in the membrane-extracted fraction (Fig. 4.7A). As receptors, these proteins are primarily expressed on the plasma membrane but would also be expected to be embedded in membranes when moving through the secretory pathway. Next, we looked at Beta-tubulin III (Tubb3), a neuronal microtubule protein that provides cytoskeletal support. Tubb3 was primarily expressed in the cytosolic fraction, although there was a small amount also found in the membrane fraction, which is not unexpected since it interacts with membrane-associated proteins and is found in the mitochondrial membrane of certain cells (Parker et al., 2022; Radwitz et al., 2022). Lastly, we examined Golgi matrix protein 130 (GM130) and Sarco/Endoplasmic Reticulum Calcium ATPase 2 (SERCA2), membrane proteins expressed in the Golgi and ER, respectively. Both markers were enriched in the membrane fraction (Fig. 4.7A). This experiment confirms that our membrane extraction of trigeminal ganglia isolated membrane-bound and membrane-associated proteins from both the plasma membrane and membranes of organelles.

We next performed an N-cadherin immunoprecipitation on the membrane-extracted fraction followed by immunoblotting for TrkA. We observed an interaction between N-cadherin and the 107, 137, and 223 kD forms of TrkA (Fig. 4.7B). Given data on the partially glycosylated form of TrkA (107 kD in our experiments) from PC12 cells as well as the results from our Brefeldin A treatment (accumulation of the 107/98 kD bands after blocking ER to Golgi transport, Fig. 4.4), these results suggest that the 107 kD TrkA-N-cadherin interaction is occurring on intracellular membranes. Because sialic residues are added to TrkA in the Golgi, it is possible that the interaction between N-cadherin and the 137 kD band of TrkA is occurring in the Golgi, at the plasma membrane, or in both locations. This experiment demonstrates that the interactions between N-cadherin and TrkA are occurring on neuronal membranes and are likely happening both intracellularly and at the plasma membrane.

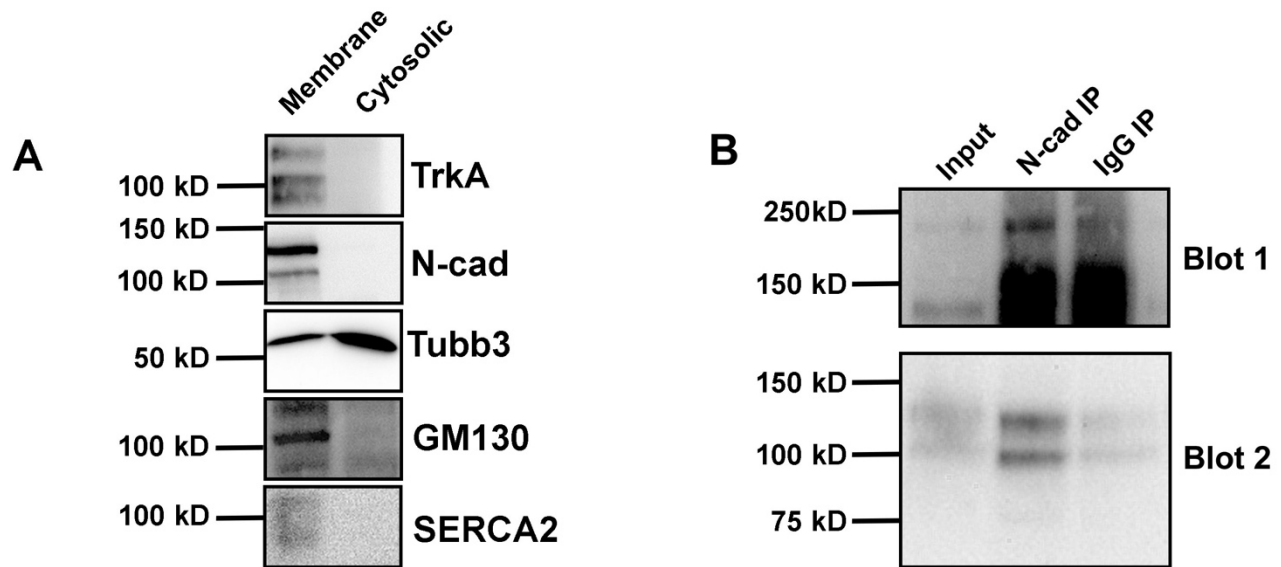


Fig. 4.5 TrkA and N-cadherin interact on neuronal membranes.

(A) Immunoblots of E6.5/HH28-30 trigeminal ganglia lysate following Mem-PER membrane extraction protocol for TrkA, N-cadherin, Tubb3, GM130 (Golgi protein), and SERCA2 (ER protein) using membrane-extracted and cytosolic-extracted protein fractions. (B) N-cadherin-TrkA co-immunoprecipitation on membrane-extracted fraction. Input contains 10% of lysate loaded in immunoprecipitation lanes. The top blot image is from one experiment using a high exposure and the bottom blot image is from a separate experiment with a lower exposure. IP, immunoprecipitation, IgG, Immunoglobulin G (N=3).

4.5 Discussion

Although post-translational modifications of TrkA have been thoroughly examined in cell lines, whether these same modifications occur *in vivo* remain poorly understood. In this study, we explored the glycosylation of TrkA in chick trigeminal neurons. Our experiments led us to confirm that the 107 kD and 137 kD bands commonly associated with TrkA in the literature are an N-linked partially glycosylated form and a N-linked and sialylated fully glycosylated form, respectively. Through our experiments, we also observed multiple lower molecular weight forms of TrkA, as well as changes to TrkA after probing for O-linked glycans. Using this characterization, we next examined interactions between TrkA and the cell adhesion molecule N-cadherin and found that N-cadherin interacts with both partially and fully glycosylated versions of TrkA, as well as a higher and two lower molecular weight forms of TrkA. These interactions were observed in both trigeminal neuron cell bodies and axons, and on neuronal plasma membranes and intracellular membranes, highlighting a previously uncharacterized interaction between neurotrophic signaling receptors and cell adhesion that underlies trigeminal ganglion neurodevelopment.

Maturation of chick TrkA involves multiple glycan modifications

To evaluate the identity of the TrkA bands observed in chick trigeminal ganglia, we first performed a series of experiments centered around perturbing or enriching for glycan residues on TrkA. Treatment of trigeminal ganglia lysate with PNGase F, which cleaves the glycosidic bond in N-linked glycoproteins, yielded one strong 88 kD band, confirming that the 93 kD, 107 kD, and 137 kD TrkA bands observed in the input lane are all N-linked glycosylated. In addition, we observed a 67 kD and a 56 kD band, likely the 76 kD and 59 kD TrkA bands observed prior to treatment, respectively. Whether these additional bands are intermediate forms of TrkA as it

undergoes glycosylation or cleaved fragments of TrkA is not known. TrkA, along with other RTKs, is proteolytically processed to generate a soluble ectodomain fragment that is approximately 100 kD and a membrane-bound fragment of 41 kD (Cabrera et al., 1996; Díaz-Rodríguez et al., 1999). Based on this, it is possible that the 93 kD TrkA we observed is the soluble ectodomain of TrkA; however, caveats exist with this interpretation. After cleavage, the shed ectodomain diffuses away from the plasma membrane. For our experiments to detect this TrkA fragment, it would need to remain closely associated with the trigeminal ganglion. This is plausible, though, given how compact the neurons are at this stage in development and the potential for “trapping” the TrkA shed ectodomain in the extracellular matrix and mesenchyme surrounding the ganglion. Although intracellular cleavage fragments of TrkA remain poorly characterized, evidence of such processing has been reported in the human receptor (Merilahti et al., 2017; Merilahti & Elenius, 2019). However, we do not believe any of the TrkA bands observed in our experiments are intracellular fragments since our TrkA antibody recognizes an epitope in the extracellular domain.

Interestingly, the 53 kD band was the only form of TrkA that did not change after PNGase F treatment, suggesting that it lacked N-linked glycans. Neuraminidase treatment altered only the 137 kD and 93 kD bands, both of which endured a slight reduction in molecular weight, indicating that these are the only sialylated forms of TrkA. This result was surprising, because if sialylation were the only additional modification occurring to transform 107 kD TrkA to the 137 kD form, a total collapse of the 137 kD band into the 107 kD band after neuraminidase treatment would be expected. However, this was not the case, suggesting other modifications, such as O-linked glycosylation, could be occurring in addition to sialylation to generate this 137 kDa form. Alternatively, it is possible that the neuraminidase enzyme might not have access to all the sialic

acid moieties in TrkA due to the complexity of the protein structure, or our treatment time was insufficient to remove every sialic acid. Even with these considerations, our results mirror those observed for TrkA after neuraminidase treatment in PC12 cells, where the 137 kD band also was not completely reduced to the 107 kD band (Jullien et al., 2002).

We next sought to test whether O-linked glycosylation was present on TrkA. While the TrkA bands observed after O-glycosidase + neuraminidase treatment were almost identical to neuraminidase treatment alone, surprisingly, a very prominent 177 kD band was also evident after O-glycosidase + neuraminidase treatment. This band likely arises from enzymatic alterations to the 223 kD form of TrkA that we sometimes observe in the input control (Supplemental Fig. 1) as well as in the N-cadherin-TrkA co-immunoprecipitation. Based on the size of this band in combination with what is reported in the literature (Hartman et al., 1992), it is possible that this is a NGF-TrkA complex containing Core 1 and 3 O-glycans. The PNGase F + neuraminidase treatment yielded the same result as the PNGase F only treatment, with one pronounced band at 88 kD along with lower molecular weight bands at 67 kD, 56 kD, and 53 kD. This result indicates that TrkA is heavily N-linked glycosylated, and N-glycans serve as the base upon which terminal glycans, including sialic acids, are built. While the treatment with all three enzymes yielded bands observed in other treatments, interestingly, we detected a 52 kD band that was not found in any other condition. One possible explanation is that the combined enzymatic treatment enhanced glycan accessibility, allowing the enzymes to act with greater efficiency. This form of TrkA might be the nascent protein, or a very slightly modified form of TrkA shortly after synthesis. Given the dynamics of protein translation, it is possible that we were unable to capture the truly unmodified form of TrkA as often these modifications are added as proteins are being translated (Helenius & Aebersold, 2001). Furthermore, our incubations may not have given the enzymes sufficient time to

cleave all the residues from TrkA. However, we do not think this is the case since previous work performed PNGase F treatment for up to 36 hours and still did not uncover the “core” TrkA protein (Watson et al., 1999). It is equally plausible that other types of post-translational modifications are present on these TrkA forms that are unrelated to glycosylation. In addition to the importance of phosphorylation on TrkA function, ubiquitination regulates TrkA and therefore could be contributing to these changes in molecular weight (Georgieva et al., 2011; Yu et al., 2014). Interestingly, our input control and mock control samples yielded slightly different results, with the 223 kD form of TrkA only present in the former. This could be due to the presence of additional reducing agents (SDS and DTT) in the latter’s denaturation buffer, which, along with the Laemmli buffer, cause this higher molecular weight complex to come apart.

To further parse out these different glycans, we performed lectin binding assays. While ConA binds to alpha-linked mannose residues, WGA recognizes N-acetyl- glucosamine groups and sialic acids. Therefore, we hypothesized that ConA and WGA enrichment would enhance the 107 kD, N-linked glycosylated and 137 kD sialylated TrkA bands, respectively. Interestingly, both bands were enriched with both treatments. Although this is in contrast to similar experiments performed in PC12 cells (Jullien et al., 2002), this difference can be explained by the lysate source. The embryonic environment is more complex and heterogenous than cell lines; therefore, it is possible that TrkA glycosylation within the embryo contains additional and/or different glycans. Next, we used PNA, which binds Galactosyl- β (1-3)-N-acetyl-galactosamine, a type of O-linked glycan, based on other work that found O-linked glycans on TrkA (N.-Y. Lin et al., 2022). Surprisingly, a 117 kD TrkA band was enriched after PNA treatment. Because this band was not observed in these other treatments and did not appear in our enzyme assays, we believe that this is an intermediate version of TrkA that is expressed at low levels. Surprisingly, ConA, WGA, and

PNA all enriched the 177 kD band observed after O-glycosidase treatment. Thus, this band contains both N- and O-linked glycans. Although this 177 kD band also appeared when all three enzymes (PNGase F, neuraminidase, and O-glycosidase) were combined, it is possible that the enzymatic digestion time was insufficient, or that this form of TrkA contains complex secondary structures that make it difficult for PNGase F and neuraminidase to properly access all moieties. Another explanation is that the 177 kD form of TrkA contains Core 2 and Core 4 O-glycans, which do not get cut by O-glycosidase. A more thorough analysis of these TrkA glycoforms through mass spectrometry would be beneficial to further classify the profile of these bands.

In concert with these experiments, we treated trigeminal neuronal cultures with inhibitors of the secretory pathway to define how these perturbations impacted the processing of TrkA. Although the molecular weights observed for TrkA in this experiment (98 kD and 122 kD) were slightly different than those observed *in vivo* and our other *ex vivo* experiments (107 kD and 137 kD), we believe they represent the same TrkA glycoforms and are just disparities due to the nature of SDS-PAGE. Monensin inhibits transport out of the Golgi by disrupting ion gradients. After immunostaining, we noted a near total accumulation of TrkA in a perinuclear location, which we expect is the Golgi. While little is known about TrkA sialylation, sialic acids are added to proteins as they move through the Golgi stacks (Zhu et al., 2024). Therefore, we would not expect the fully glycosylated 137 kD form of TrkA to be observed in the Monensin-treated group, but rather only the 107 kD form that is accumulating within the Golgi apparatus. Brefeldin A disrupts anterograde transport from the ER to the Golgi and causes disruption of the Golgi complex (Reaves & Banting, 1992). This treatment led to diffuse TrkA staining throughout the soma, and a robust 107 kD band after immunoblotting, suggesting that, in chick trigeminal neurons, the 107 kD partially glycosylated form of TrkA is in the ER, and further confirming that sialylation occurs largely

within the Golgi. The diffuse staining of TrkA likely corresponds to the partially glycosylated form being dispersed throughout the ER of the cell, and similar results have been observed after Brefeldin A treatment in other types of neurons (Jareb & Banker, 1997). Lastly, Tunicamycin treatment, which prevents all N-linked glycosylation from occurring, led to accumulation of TrkA throughout the soma cytoplasm, and a near complete loss of both the 107 kD and 137 kD TrkA bands. Tunicamycin induces ER stress, protein misfolding, and eventual cell death (Heifetz et al., 1979). In keeping with this, TrkA immunostaining appears dim and punctate, suggesting unhealthy cells and protein processing. While the 107 kD and 137 kD bands were diminished after Tunicamycin treatment, very interestingly, that treatment group was the only condition in which a lower molecular weight band of 80 kD accumulates, supporting the notion that this could be an “immature” form of TrkA that possesses little to no glycosylation. These findings could reflect the differences noted for TrkA immunoblotting banding patterns *in vivo* and *ex vivo* and/or indicate that the Tunicamycin treatment time was not sufficient to remove all N-glycans and reveal the true core protein, especially since the 137 and 107 kD bands are still present. One main limitation of this experiment is the lack of available organelle markers compatible with immunostaining of chick tissue. Having clear markers to label organelles would be an excellent addition to these studies to verify the subcellular location of TrkA accumulation within the cells.

TrkA interacts with N-cadherin dynamically in different compartments of trigeminal neurons

We next sought to examine the coexpression of TrkA with N-cadherin in E5.5/HH28 chick embryos and found that both *in vivo* and *ex vivo*, the two colocalized along the plasma membrane of trigeminal neurons. Although it was difficult to detect intracellular colocalization via immunostaining in our *in vivo* samples due to the compact nature of the trigeminal ganglion at this stage of development, we did observe intracellular colocalization of TrkA and N-cadherin *ex vivo*,

likely within vesicles and in perinuclear structures. Given that we observed TrkA and N-cadherin colocalization in both the cell bodies and along the axons, we chose to explore whether these two proteins were interacting in both compartments. Through co-immunoprecipitation assays, we found that N-cadherin interacts with the 107 kD and 137 kD form of TrkA in cell bodies but only the 137 kD form of TrkA in axons. TrkA synthesis occurs in the cell bodies of neurons as it progresses through the secretory pathway to transform from an immature protein into a fully glycosylated receptor. Only then does it undergo anterograde trafficking through the axon to be inserted into the axonal plasma membrane, where it acts as a signaling receptor for NGF. Thus, the absence of the 107 kD TrkA band from both the input and IP lanes of our axon sample is not surprising and suggests that this band is a partially glycosylated form of TrkA moving through the secretory pathway within the cell bodies. Because mature TrkA interacts with NGF on the axon terminals, an interaction between the 137 kD mature form of TrkA and N-cadherin in the cell bodies is likely occurring on the plasma membrane or within vesicles either traveling anterogradely or retrogradely from axons. Experiments in rodent sympathetic ganglia have shown that transcytosis can cause the mature TrkA receptor to be endocytosed from the surface of cell bodies and anterogradely trafficked to the axon surface (Ascaño et al., 2009; Connor et al., 2023; Yamashita et al., 2017). It is possible that this process is also occurring in chick sensory ganglia. In both compartment immunoprecipitations, we observed a larger molecular weight band at 223 kD in addition to two smaller 76 kD and 59 kD bands. Previous studies noted a band size of 220 kD in PC12 cells that likely represents a NGF-TrkA protein complex based on radiolabeling (Hartman et al., 1992). As such, the 223 kD band in our experiments could represent an NGF-bound TrkA-N-cadherin complex, while the lower molecular weight bands could be differently

glycosylated forms of TrkA interacting with N-cadherin as they move through the secretory pathway.

While our co-immunoprecipitation experiments confirm an interaction between N-cadherin and multiple different forms of TrkA, whether they are directly interacting extracellularly, intracellularly, or indirectly via a complex remains unanswered. Cadherin-RTK interactions contribute to cellular processes underlying both development and disease, which have given insight into how these receptors regulate each other. Typically, cadherins and RTKs directly interact via their extracellular domains, as noted in E-cadherin-EGFR and N-cadherin-FGFR interactions (Andl & Rustgi, 2005; T. Nguyen & Mège, 2016b; Qian et al., 2004a). Moreover, in some cases, the binding of the cadherin to the RTK limits the ability of ligand binding (Qian et al., 2004a; Blaschuk, 2025), although we do not believe this to be the case in our experiments given the presence of the 223 kD TrkA band that is purported to possess NGF. Conversely, N-cadherin is also capable of preventing FGFR from getting internalized after ligand binding, keeping it constitutively active at the membrane to promote metastasis (Suyama et al., 2002). Another possibility is that N-cadherin and TrkA are not interacting directly with each other and are instead forming a complex through adaptor proteins. Two potential candidates for this are Shc and β -catenin, as demonstrated in cell lines. Shc, a common adaptor protein for neurotrophic signaling, binds the C-terminus of N-cadherin (Y. Xu et al., 1997) while β -catenin, a crucial connector of cadherins to the actin cytoskeleton, interacts with Trk receptors (David et al., 2008). Future studies would aid in addressing whether the interaction between N-cadherin and TrkA is direct or indirect.

TrkA and N-cadherin interactions occur both at the cell membrane and intracellularly

We lastly sought to explore where the interactions between N-cadherin and TrkA seen from Fig. 4 were occurring within the trigeminal neurons. To test this, we separated all membrane-

bound and membrane-associated proteins from cytosolic proteins and performed an immunoprecipitation for N-cadherin followed by immunoblotting for TrkA. Both the partially and fully glycosylated forms of TrkA were found to interact with N-cadherin in the membrane fraction, as well as the 223 kD band which we hypothesize to be a NGF-TrkA complex. Given the cumulative knowledge surrounding cadherin-RTK interactions, and how they commonly regulate each other's activity at the plasma membrane, it is not surprising to see the functional form of TrkA interacting with N-cadherin, either on the plasma membrane or within other membrane-possessing structures like vesicles (e.g., signaling endosomes) (Harrington & Ginty, 2013; Howe et al., 2001; Sharma et al., 2010). Furthermore, if the 223 kD band we observe is truly a NGF-TrkA complex that associates with N-cadherin, this interaction would likely be found at the plasma membrane or on the membrane of an internalized vesicle. Strikingly, we observed that the 107 kD, partially glycosylated form of TrkA also interacts with N-cadherin. Because only the 137 kD form of TrkA reaches the plasma membrane, this interaction with the 107 kD form likely occurs intracellularly, either on a membrane of the ER, Golgi, or within a transport vesicle. We can also not rule out that the interaction between the 137 kD form of TrkA and N-cadherin solely occurs on the plasma membrane. This interaction could also be taking place on the Golgi membrane after TrkA has been fully modified, or on a vesicle traveling to the plasma membrane. While there is very limited knowledge surrounding intracellular cadherin-RTK interactions, ER to Golgi transport of N-cadherin-FGFR complexes has been demonstrated (Nakamura et al., 2008). Thus, a similar phenomenon could be occurring between N-cadherin and TrkA as they travel through the secretory pathway. Future experiments examining the subcellular location of this interaction would give more insight into its functional role.

Conclusion

In this study, we identified different glycoforms of the neurotrophic receptor, TrkA, in chick trigeminal sensory neurons and demonstrate that these forms of TrkA interact with N-cadherin on the membranes of neurons. These results contribute to our general understanding of how post-translational modifications and membrane interactions regulate TrkA localization during sensory development. Given the integral roles that both TrkA and N-cadherin play during neuronal development, their interaction likely contributes to the coordinated assembly of the trigeminal ganglion. As aberrant RTK and cadherin signaling are increasingly becoming recognized in pathological conditions, and subsequently contribute to altered cell fate and proliferation, understanding how these pathways intersect during normal development can provide important knowledge on how their dysregulation contributes to diseases. Future studies assessing how N-cadherin modulates TrkA trafficking, signaling, and localization at the membrane will clarify the mechanisms behind adhesion-dependent signaling that shapes the sensory nervous system.

Chapter 5: Conclusions and Future Directions

5.1 Summary

Although the cells that give rise to sensory ganglia have been known for decades, the molecular network that coordinates the generation of these complex structures remains largely unexplored. The overarching goal of this dissertation sought to investigate the role of cadherins during trigeminal gangliogenesis, with a particular focus on N-cadherin and how its interaction with other molecules contributes to the assembly and maturation of the trigeminal ganglion. To address this, we examined N-cadherin function over multiple developmental stages. During the earliest stages, where neural crest cells and placode-derived neurons first coalesce and adhere to form a single structure, we characterized cadherin interactions between Cadherin-7-expressing neural crest cells and N-cadherin-expressing placode-derived neurons. Through this work, we discovered that Cadherin-7 and N-cadherin engage in heterophilic cadherin interactions which likely mediate the earliest stages of gangliogenesis as these two cell populations are beginning to intermingle and condense with one other. As the trigeminal ganglion condenses, extends axons, and establishes connections with target tissues, we explored how depletion of N-cadherin from placode-derived neurons affected overall trigeminal ganglion morphology and axon outgrowth. This research led to the conclusion that N-cadherin has long-standing importance during gangliogenesis. The loss of N-cadherin significantly impacted trigeminal ganglion size during multiple stages of development, reduced axon outgrowth as observed by impacted target innervation, and led to a reduction in the number of neurites produced by neural crest-derived neurons, showing a previously unknown non-cell autonomous role of N-cadherin in placode-derived neurons on neural crest-derived neurons. Lastly, at later stages when trigeminal neurons are beginning to specify into subclasses that relay distinct sensations, we investigated interactions

between N-cadherin and TrkA, an RTK. These experiments showed that N-cadherin and TrkA physically interact in both cell bodies and axons of trigeminal neurons, and these interactions likely take place both at the cell membrane and on the membrane of organelles as these proteins move through the secretory pathway. While cadherin-RTK interactions have been shown in certain contexts such as N-cadherin-PDGFR interactions in neural crest cell migration (Bahm et al., 2017), Cadherin-11-PDGFR interactions in mesenchymal cells (Madarampalli et al., 2019b), and E-cadherin-EGFR interactions in epithelial cells (Hoschuetzky et al., 1994), interactions between N-cadherin and TrkA have never been demonstrated before, uncovering a new cellular interaction within trigeminal neurons. Taken together, the findings from this dissertation provide a multi-stage analysis of N-cadherin during trigeminal gangliogenesis and add to our cumulative knowledge of the molecules underlying sensory neurodevelopment.

5.2 Cadherin-7 and N-cadherin engage in heterophilic cadherin interactions during early gangliogenesis

In Chapter 2, we sought to investigate whether heterophilic cadherin interactions were mediating coalescence between neural crest cells and placode-derived neurons. Previous parallel studies examining knockdown of Cadherin-7 in neural crest cells and N-cadherin in placode-derived neurons displayed similar phenotypes – perturbation to initial ganglion condensation (Shiau & Bronner-Fraser, 2009a; C. Wu & Taneyhill, 2019). The resemblance between the two phenotypes alluded to the possibility that Cadherin-7 and N-cadherin interactions were mediating early gangliogenesis. Using biochemical approaches and the GRASP split GFP method, we demonstrated that these two cadherins biochemically interact within trigeminal ganglia lysate, and these physical interactions are detectable both *in vitro* and *in vivo*. Although this work established

a foundational understanding of a heterophilic cadherin interaction mediating early gangliogenesis and of utilizing GRASP in the chick embryo, additional experiments would be of great benefit to further demonstrate the relevance of this interaction.

One of the main challenges in this work was how infrequently *trans* GFP-puncta were observed *in vivo*. We believe this is due to a combination of the inability of neural crest and placode cells to highly express these split GFP fusion constructs, and to properly traffic and target them to a location on the plasma membrane where they are then in close enough proximity to their compatible split GFP-plasmid to reconstitute GFP. Given the small size of the observed reconstituted GFP puncta within the tissue, it is possible that a higher resolution microscope would have detected them more readily. Alternatively, sequentially electroporated trigeminal ganglia explant cultures might also yield more robust results, as explants allow us to examine cellular interactions at a single-cell resolution.

Since the release of this publication, unpublished data from our lab has indicated that the majority of neural crest cells that differentiate into neurons of the trigeminal ganglion delaminate between 6-8ss. Given that the neural crest electroporations were performed at earlier stages of development in this work (2-4ss), it is highly unlikely that the interactions observed between Cadherin-7 and N-cadherin were occurring between neural crest cells that would give rise to neurons. Instead, these Cadherin-7-expressing neural crest cells are more likely precursors of glial cells, the other neural crest-derived source found in the trigeminal ganglion. However, because the time between the 2-4ss and 6-8ss electroporation is relatively short (approximately 6 hours), it is possible that the electroporated plasmid would still be present in premigratory neural crest cells undergoing EMT and beginning to migrate at the 6-8ss, even with cell division. Therefore, it cannot be fully ruled out that some of these Cadherin-7-expressing neural crest cells are neuronal

precursors. Given this, it would be interesting to assess whether these heterophilic interactions occurred differently based on the fate of the neural crest cell. More specifically, is this a transient interaction that occurs only during early gangliogenesis between undifferentiated neural crest cells and placode cell-derived neurons? Or is this interaction sustained throughout development, and occurs after differentiation between Cadherin-7-expressing glial cells and N-cadherin-expressing neurons?

While certain pathways such as Slit/Robo, Wnt, and Neuropilin/Semaphorin have been established as regulators of early gangliogenesis, understanding their role on Cadherin-7 expression within neural crest cells and N-cadherin expression within placode cell-derived neurons is less understood. Aside from Robo2 acting on N-cadherin post-translationally in placode-derived neurons (Shiau & Bronner-Fraser, 2009c), very little is known about how upstream signaling controls the expression and/or activity of these two cadherins and trigeminal ganglion coalescence. Experiments perturbing these known regulators of gangliogenesis and observing their impact on cadherin expression within neural crest and placode cells, and further, whether the amount GRASP puncta changed in response, would give additional insight into the upstream signaling pathways that modulate cadherin expression and interactions underlying early gangliogenesis. If these pathways do regulate cadherin expression, another possible experiment would be to temporally dissect when these signaling pathways are acting on them. To test this, perturbations of these signaling pathways could be performed at early developmental stages, prior to when GRASP puncta were observed *in vivo* (before HH13/14), and later in development after GRASP puncta were noted (after HH13/14). If GFP puncta formation is diminished after the early stage perturbations, this would indicate that these signaling pathways serve to establish heterophilic cadherin interactions. However, if perturbations to these signaling pathways are performed later,

and GFP puncta disappear, this would suggest that these pathways act to maintain heterophilic cadherin interactions. Answering these questions would add to our general understanding of how the trigeminal ganglion is built.

5.3 N-cadherin plays a role in trigeminal ganglion neurodevelopment and axon outgrowth to targets

In Chapter 3, we aimed to understand the importance of N-cadherin-expressing placode-derived neurons on gangliogenesis throughout multiple embryonic stages. While previous studies had concluded that reducing N-cadherin from placode-derived neurons impacted the ability of placodal neurons from properly condensing during early stages of development, these studies were limited to one stage grouping, and did not explore whether this early reduction of N-cadherin led to sustained morphological and functional defects of the trigeminal ganglion (Shiau & Bronner-Fraser, 2009a). Building upon these studies, we discovered that N-cadherin depletion from placode cells has long-term effects on trigeminal ganglion morphology and axon outgrowth, including developmental stages in which neural crest cells are differentiating into neurons. This observation next led us to explore whether N-cadherin-mediated adhesion was contributing to these phenotypes. Using a function-blocking antibody against the extracellular domain of N-cadherin, we observed decreased axon outgrowth of both placode- and neural crest-derived neurons *in vitro*, leading to the conclusion that N-cadherin-mediated adhesion was needed for both cell types to extend axons to their targets properly. These *in vitro* experiments indicate that our *in vivo* phenotypes are at least in part due to disruptions to N-cadherin-mediated adhesion, and suggest that N-cadherin-expressing placode-derived neurons non-cell autonomously regulate neurite and subsequent axon production in neural crest-derived neurons. Our work demonstrated decreased

levels of β -catenin, providing insight into the mechanisms underlying the observed phenotypes; however, additional experiments would be beneficial in understanding how N-cadherin within placode cells regulates axon outgrowth of both cell populations within the trigeminal ganglion.

One of the most intriguing phenotypes observed with the experiments from Chapter 3 are the persistent morphological defects to the trigeminal ganglion and nerve branches at later developmental stages, where neural crest cells have started differentiating into N-cadherin-expressing neurons after N-cadherin knockdown from placode cells. This suggests that there is a non-cell autonomous role of placode cell-derived neurons on neural crest-derived neurons. While our sequential electroporation explant experiments with GFP-labeled neural crest cells and N-cadherin MO-labeled placode cells showed diminished neural crest-derived neurite production *in vitro*, suggesting our *in vivo* phenotypes are due to this, we were unable confirm this definitively *in vivo* due to missing experiments needed to establish a causal link. Multiple technical challenges limited our ability to perform these experiments and grow the embryos out to later stages in development. Performing two electroporations and introducing two synthetic constructs during embryogenesis imposes significant stress on the developing embryo, resulting in a high rate of lethality. This issue was compounded by the need to re-incubate the embryos for more than one week to reach stages at which neural crest-derived neurons have been reported *in vivo* (D'Amico-Martel & Noden, 1980; D'amico-Martel & Noden, 1983), further reducing the number of surviving embryos available for analysis. Additionally, the timing of when the neural crest cells that differentiate into trigeminal neurons delaminate from the neural tube remains poorly defined, complicating our ability to target these cells efficiently. While unpublished data from our lab indicate that electroporation at the 6-8ss labels the most neural crest cells fated to become trigeminal neurons, there was still a high level of variability in the number of GFP-positive neurons

labeled. Performing a systematic electroporation time course across early somite stages, followed by quantification of labeling efficiency, would help identify the optimal stage for targeting these trigeminal neural crest cells. Establishing this would remove one major source of variability and allow future experiments to focus primarily on overcoming the stress and lethality associated with the sequential electroporation technique.

Placode cell-derived neurons are present in the trigeminal ganglion earlier than neural crest-derived neurons, suggesting that a subpopulation of them functions as pioneer neurons (Moody et al., 1989). Although our results support this notion, performing sequential electroporations to label both cell populations with fluorescent reporters and analyzing multiple developmental timepoints during gangliogenesis would definitively prove this. This type of experiment would clarify how many trigeminal neurons act as pioneers. Building off this, findings from Chapter 3, Fig. 5, indicate that the lateral and medial nasal nerve branches of OpV respond differently to N-cadherin knockdown. While we did not explore this in detail, it is possible that the relative contributions of neural crest- and placode cell-derived neurons differs between these two nerve branches. If one branch contains proportionally more placode cell-derived neurons, and thus a greater loss N-cadherin, this could explain why we observed different phenotypes. However, until dual cell labeling experiments to map the cellular composition of each branch are performed, this remains speculative.

We concluded that a subset of placode cell-derived neurons likely serves as pioneers based on our *in vitro* function-blocking experiments. When placode cells were labeled with RFP, a subpopulation continued to extend axons even after blocking N-cadherin-mediated adhesion. Because pioneer neurons generally rely more heavily on ECM interactions, while follower neurons depend more heavily on cell adhesion, this behavior is consistent with pioneer-like properties.

However, we did not directly demonstrate that the RFP-positive cells extending axons after blocking N-cadherin mediated adhesion were pioneer neurons. To test this more rigorously, additional function-blocking experiments targeting integrins could be performed given their known interactions with the ECM and role in guiding regenerated and/or pioneer neurons (T. M. Gomez et al., 1996; Lein et al., 1991; Moreland & Poulain, 2022; Myers et al., 2011; Tomaselli et al., 1993; Varnum-Finney et al., 1995; Venstrom & Reichardt, 1995). Trigeminal neurons express integrins that bind fibronectin, a major component of the ECM and the substrate used in our explant assays. If integrin-mediated interactions were needed for axon extension of this subpopulation, then simultaneous inhibition of integrins and N-cadherin might lead to a total loss of trigeminal axon outgrowth. This would support the idea that the N-cadherin-independent subpopulation of placode cell-derived neurons relies primarily on ECM-based interactions rather than cell-cell adhesion, further supporting their identity as pioneers.

While our experiments demonstrated a non-cell autonomous role for N-cadherin within placodal neurons on neural crest-derived neurite production *in vitro* and decreased levels of β -catenin within neural crest-derived neurons, the mechanism behind this remains unclear. Loss of N-cadherin from the membrane of placodal neurons may destabilize the amount of β -catenin anchored at the cell membrane, causing it to get degraded more rapidly and accounting for the reduced levels we observed. However, providing a direct link between the reduced levels of β -catenin and impaired neurite production in neural crest-derived neurons would solidify this connection. Future experiments could address this question in multiple ways. First, rescuing β -catenin levels after N-cadherin MO electroporation and assessing whether neurite production in MO-treated explants is comparable to controls would confirm β -catenin's role in this process. Although our experiments qualitatively saw less β -catenin at the neuronal cell membrane, we did

not determine whether this loss is restricted to the membrane or is apparent across the entire cell. To address this, membrane-bound and cytosolic fractions of β -catenin could be separated and analyzed by immunoblotting in MO- and control trigeminal ganglia, providing direct evidence of subcellular changes following N-cadherin knockdown. Finally, it would be important to distinguish whether β -catenin's effect on neurite production arises solely from its structural role at the membrane or also from its signaling function in the Wnt pathway. Using a Wnt pathway inhibitor would specifically disrupt β -catenin-mediated transcription without disrupting its adhesion-dependent roles at the cell membrane. Assessing neurite outgrowth under these conditions would clarify the contribution of β -catenin to these phenotypes. Together, these experiments would provide strong mechanistic insight into how N-cadherin knockdown in placode-derived neurons impacts neurite production in neural crest-derived neurons.

5.4 N-cadherin interacts with multiple glycoforms of TrkA in trigeminal neurons

In Chapter 4, we explored interactions between N-cadherin and the neurotrophic factor receptor, TrkA. While performing experiments from Chapter 3 (Fig. 3.4), we observed abnormal distribution of TrkA in trigeminal neurons after N-cadherin knockdown. More thorough analysis of this phenotype led us to hypothesize that the processing on TrkA might be impacted after N-cadherin knockdown. Using immunohistochemistry, in control tissue, we saw TrkA along the plasma membrane and diffuse within the cytoplasm (Fig. 5.1A), while in N-cadherin MO-treated tissue, we observed a perinuclear accumulation of TrkA, which we speculated could either be the ER or the Golgi (Fig. 5.1B). After immunoblotting, we observed an increase in the partially glycosylated form of TrkA compared to the fully mature form in the N-cadherin MO-electroporated trigeminal ganglia (Fig. 5.1C). These phenotypes raised the question as to whether

N-cadherin and TrkA were regulating each other within the trigeminal ganglion, as there is evidence of cadherin-RTK interactions in other cellular contexts (Chiasson-MacKenzie & McClatchey, 2018a). However, because most information about TrkA has been reported in cell lines, we were also curious to better understand TrkA post-translational modifications during gangliogenesis, given their importance in positioning TrkA on the plasma membrane as a functional signaling receptor. In this work, we identified N-linked, O-linked, and sialic acid residues on TrkA within trigeminal neurons, and observed that multiple differentially modified forms of TrkA interact with N-cadherin. We further sought to clarify where these interactions were taking place within neurons and concluded that while different interactions occur within cell bodies and axons, all occur at the membrane and not the cytosol of neurons. Interestingly, because we observed a partially glycosylated form of TrkA interacting with N-cadherin that is not thought to be present at the plasma membrane, our results suggest that this interaction occurs on the membrane of organelles as these proteins travel through the secretory pathway. Our results are the first report of N-cadherin interacting with a neurotrophic factor receptor, and one of the few that report a cadherin-RTK interactions within the cell. Although our work has uncovered a new interaction within trigeminal neurons that likely regulates gangliogenesis, future studies are needed to uncover the functional importance of this interaction.

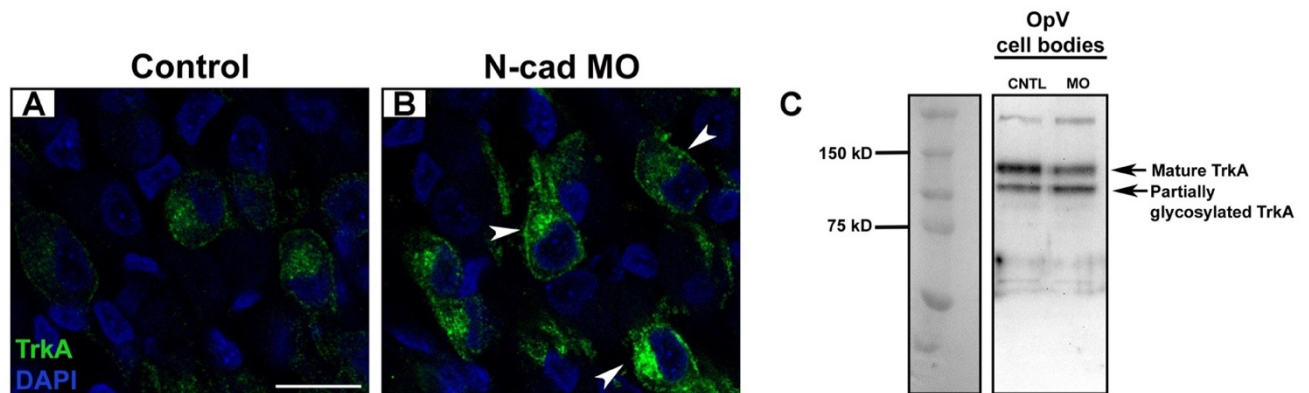


Fig 5.1 N-cadherin MO in placode cells impacts the processing of TrkA.

(A-B) Representative images of E6.5/HH28-30 trigeminal ganglion (contralateral control (A) and N-cadherin MO (B)-electroporated side) after TrkA immunostaining (1:5000) and DAPI stain to mark nuclei. Arrowheads in (B) indicate TrkA accumulation. Scale bar in (A) is 25 μ m and applies to (B). (C) Immunoblots of E6.5/HH28-30 electroporated (MO) and contralateral (CNTL) trigeminal OpV lobes for TrkA (n=1, 1:5000).

Because the partially glycosylated form of TrkA interacts with N-cadherin, we inferred that this interaction is occurring within the membrane of organelles. Determining the precise subcellular location of this interaction would clarify its functional significance. To do this, sucrose gradient fractionation, which separates subcellular components based on their density, if done stringently enough, could separate the ER, Golgi, plasma membrane, and endosomes from one another. Preliminary unpublished data from our lab demonstrate that discontinuous sucrose gradient centrifugation can enrich for different organelles from trigeminal ganglia (Fig. 5.2). However, some organelles have very similar densities, making it very difficult to separate them clearly (Table 5.1). Without further refining the sucrose gradient to enrich for a single organelle, identifying which organelle(s) contain the N-cadherin-TrkA interaction remains challenging. A proximity ligation assay (PLA) would provide a complimentary approach, allowing for the visualization of the N-cadherin-TrkA interaction at subcellular resolution and potentially identifying in which organelle the interaction occurs. Unfortunately, the limited availability of organelle-specific antibodies available for chick tissue currently decreases the feasibility of PLA.

Co-IPs performed on sucrose gradient fractions would not only reveal where the interaction occurs, but which forms of TrkA are interacting with N-cadherin. If the partially glycosylated form co-IPs with N-cadherin within the ER or the Golgi fractions, it would further validate that this interaction occurs before TrkA reaches the plasma membrane. Conversely, we would expect the fully glycosylated form to interact with N-cadherin in the plasma membrane fraction, but interactions in the Golgi or endosome fraction are also plausible. Because TrkA fully matures within the Golgi and may be trafficked with N-cadherin during internalization or recycling, interactions with the fully glycosylated forms could be evident there. If an endosomal interaction is detected, distinguishing among early, late, and recycling endosomes would be highly

informative. Although we performed co-IPs on the sucrose gradient fractions, we were unable to detect an interaction between N-cadherin and TrkA (Fig. 5.3). However, several technical challenges likely contributed to our inability to detect this interaction. The high-speed centrifugation that occurs during sucrose gradients imposes mechanical stress that may disrupt transient or weak protein-protein interactions (Caudron-Herger et al., 2020). Cross-linking the tissue prior to this experiment might help with this potential issue (Klockenbusch & Kast, 2010). Second, our standard co-IP requires 250 µg of protein, but each step of the gradient separation, fraction isolation, and lysis drastically reduces protein yield. Therefore, it is possible that substantially more starting material is needed to detect this interaction reliably.

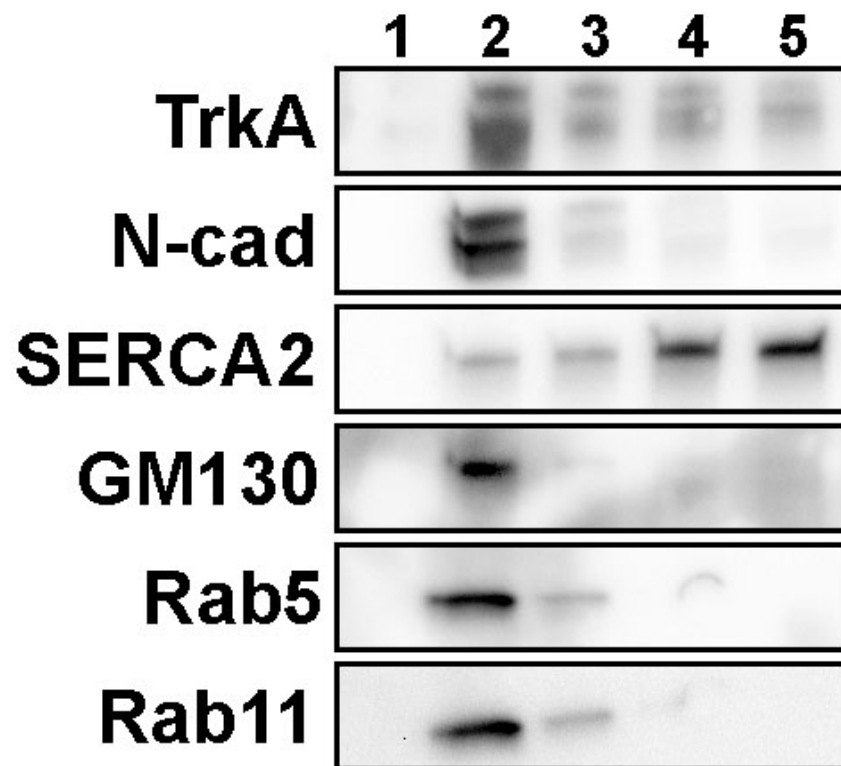


Fig. 5.2 Discontinuous sucrose gradient fractionation enriches organelles from trigeminal ganglia in different fractions.

Immunoblots of E6.5/HH28-30 trigeminal ganglia sucrose gradient density fractions (1-5) for antibodies specific to different organelles. TrkA (1:5000) and N-cad (1:100): neuronal markers, SERCA2 (1:100): ER, GM130 (1:100): Golgi, Rab5 (1:100): early endosome, Rab11 (1:100): recycling endosome. After isolating organelle pellets from each fraction, each pellet was resuspended in 1x Laemmli Buffer and the entire fraction was loaded on the gel. Fraction 1: 0.302 M, Fraction 2: 0.869 M, Fraction 3: 1.15M, Fraction 4: 1.29M, Fraction 5: 1.53M.

Subcellular Component	Density (g cm ⁻³) in sucrose
Nucleus	> 1.30
Nuclear membrane	1.18-1.22
Mitochondria	1.17-1.21
Mitoplast	1.14
Lysosomes	1.19-1.21
Peroxisomes	1.18-1.23
Rough ER	1.18-1.26
Smooth ER	1.06-1.15
Plasma membrane sheets	1.14-1.19
Plasma membrane vesicles	1.07-1.16
Endosomes	1.06-1.16
Golgi (intact)	1.05-1.12
Golgi (vesicle)	1.05-1.12
Sarcoplasmic reticulum	1.04-1.08

Table 5.1 Density of organelles isolated from rat liver tissue. Table adapted from (Dealtry & Rickwood, 1992)

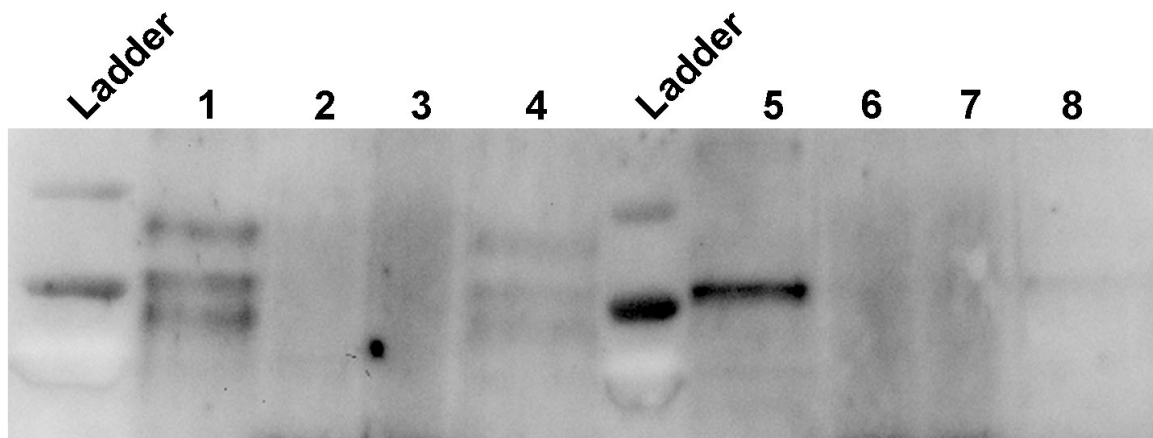


Fig. 5.3 N-cadherin-TrkA co-immunoprecipitation on sucrose gradient fractions.

Immunoblot of E6.5/HH28-30 trigeminal ganglia sucrose gradient density fractions followed by N-cadherin IP and probing for TrkA (1:5000). Fractions 2 and 3, and 4 and 5, were pooled together. Once pellets containing enriched organelles were isolated, they were resuspended in equal amounts of lysis buffer to solubilize proteins. To minimize loss of proteins, Bradford assays were not performed. Instead, IPs were done on equal volumes of solubilized proteins and input lanes contained 10% of lysate loaded in immunoprecipitation lanes. 1: Fractions 2/3 Input, 2: Fractions 2/3 N-cad IP, 3: Fractions 2/3 Rabbit IgG IP, 4: Fractions 2/3 N-cad IP flow-through, 5: Fractions 4/5 Input, 6: Fractions 4/5 N-cad IP, 7: Fractions 4/5 Rabbit IgG IP, and 8: Fractions 4/5 flow-through.

Once the location(s) of these interactions are identified, the next step is to define the functional relevance of this interaction. Do N-cadherin and TrkA regulate each other's trafficking or signaling, or are they simply co-transported through the secretory and endocytic pathways? Our preliminary experiments suggest functional regulation, as knockdown of N-cadherin disrupts TrkA distribution, consistent with impaired trafficking. Although the lack of organelle-specific antibodies for immunostaining limits experimental approaches, sucrose gradient fractionation could be run on both electroporated and control trigeminal ganglia. If TrkA becomes trapped at a particular point in the secretory pathway, gradient fractions from MO-treated samples would show an accumulation of TrkA in a specific organelle-enriched fraction. This experiment would provide direct evidence that knockdown of N-cadherin impacts the processing of TrkA. However, this type of experiment would not be easy to execute. Embryos must survive five days after the electroporations, trigeminal ganglia yields would be limited, and 5-10 mg of tissue per gradient would require extensive pooling, making this a time-consuming and labor-intensive experiment. Even so, such work could provide highly informative mechanistic insight.

Further examining the functional role of this interaction on NGF-TrkA signaling also represents an important future direction. NGF-TrkA signaling is essential for the overall health of trigeminal neurons. Our results from Chapter 3 showed that knockdown of N-cadherin reduced the outgrowth of trigeminal axons to their target tissues. Because NGF is secreted from targets and TrkA is expressed at axon terminals, a reduction in axons and subsequent TrkA raises the question of how altered ligand-receptor expression affects developing neurons. Furthermore, if N-cadherin regulates TrkA trafficking, TrkA might fail to reach axonal growth cones, further impacting signaling. High-resolution imaging of axons and growth cones could give valuable information as

to the ability of TrkA to successfully reach the plasma membrane in N-cadherin MO-treated trigeminal ganglia, establishing whether disrupted trafficking leads to impaired NGF signaling.

5.5 Significance

This dissertation demonstrates that N-cadherin plays important, multifunctional roles in building the trigeminal ganglion during multiple stages of development. Beginning at initial coalescence of the structure and following its development to stages where neurons are beginning to specialize into distinct subpopulations, this work has uncovered three major conclusions: heterophilic cadherin interactions between N-cadherin and Cadherin-7 are evident as the trigeminal ganglion begins to form, N-cadherin shapes the structure of the trigeminal ganglion and is crucial for axon outgrowth to targets, and N-cadherin engages in a novel interaction with TrkA that may regulate neurotrophic signaling.

Although this dissertation focuses on trigeminal ganglion neurodevelopment, our findings have broader implications for understanding the etiology of different human diseases. Many neurological and neurodevelopmental disorders include peripheral nervous system deficits such as hyper- or hyposensitivity to sensory stimuli as seen in autism spectrum disorder and attention deficit hyperactivity disorder, malformed cranial ganglia as noted with neurocristopathies, and chronic pain disorders such as peripheral neuropathy (Bouchatta et al., 2022; Fonseca et al., 2025; Ghanizadeh, 2011; Khan et al., 2023; Okuno & Okano, 2021; Orefice, 2020; Pilon, 2021). Mutations in both *N-cadherin* (also called *CDH2*) and *TrkA* (also called *NTRK1*) are commonly associated with these disorders, underscoring the translational relevance of the findings from this dissertation (Franco et al., 2016; Halperin et al., 2021; Y. Kim et al., 2024; László & Lele, 2022; Moraes et al., 2022). Furthermore, the discovery of a role for N-cadherin in TrkA maturation,

trafficking to the membrane, and potential regulation of its signaling with NGF support a regulatory interaction that, when perturbed, could lead to unregulated signaling and cell proliferation as observed in cancer metastasis (Qian et al., 2004b). Our data highlight how cadherin-mediated adhesion and neurotrophic signaling, which are essential during neurodevelopment, may become pathological when disrupted. Moreover, the regulatory interactions between cadherins and RTKs remain vastly unknown, and our identification of a novel interaction between N-cadherin and TrkA provides a foundation into future studies investigating how these two protein classes are hijacked in cancer progression and metastasis. Taken together, this work demonstrates that N-cadherin is more than an adhesive molecule during gangliogenesis, but actively organizes the assembly, innervation, and neuronal circuitry that gets established during development. Collectively, these results substantially expand our understanding of the molecular networks underlying sensory neurodevelopment and offer new avenues for exploring their disruption in disease.

Appendix: Exploring the subcellular location of N-cadherin-TrkA interactions.

In Chapters 4 and 5, we sought to better characterize the interactions between N-cadherin and TrkA occurring within trigeminal neurons. To this end, our goal was to perform sucrose gradient density centrifugation to isolate purified organelle fractions, followed by an N-cadherin-TrkA co-IP to determine in which fraction(s) this interaction was occurring. However, we were unable to successfully complete this experiment due to multiple technical difficulties. The experimental method used for this is described below.

Sucrose gradient

A discontinuous sucrose gradient was generated based on modifications to an existing protocol (Tarazón et al., 2020). Briefly, trigeminal ganglia were homogenized in 0.25M sucrose (in 10 mM Tris-Cl) in a 1.5 mL Eppendorf tube. Tissue was homogenized with 15 strokes of a disposable pellet pestle (Fisher) and centrifuged for 10 minutes at 1,000 xg at 4°C to pellet nuclei and cell debris. The supernatant was transferred to a new tube and resuspended in 50% volume of the original homogenization buffer and centrifuged again. Both supernatants were pooled and then centrifuged for 20 minutes at 20,627 xg at 4°C to pellet organelles. This pellet was resuspended in 206 uL 0.25M sucrose and 595 uL 2M sucrose to yield a 1.55M sucrose/organelle mixture which was carefully layered at the bottom of a 5 mL Beckman Coulter ultracentrifuge tube. 800 uL each of 1.33M sucrose, 1.20M sucrose, 1.10M sucrose, 0.77M sucrose, and 0.25 M sucrose were gently layered on top to generate the discontinuous sucrose gradient. The gradient was centrifuged for 1.5 hours at 4°C at 30,000 xg in an Optima XPN-80 Ultracentrifuge. Following initial centrifugation, 1 mL fractions were collected from the gradient and resuspended in 0.25M sucrose before undergoing centrifugation for 1 hour at 4°C at 30,000 xg to pellet organelles in individual

fractions. These pellets were either resuspended in 1× reducing Laemmli sample buffer and boiled at 99°C for 7 minutes to be separated by SDS-PAGE, or were lysed in lysis buffer (see methods in the immunoprecipitation section) and quantified by Bradford assay for subsequent experimentation.

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