

SYMPATHETIC GANGLIA FORMATION IN
THE CHICK PERIPHERAL NERVOUS SYSTEM

by

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A dissertation submitted in partial fulfillment
of the requirements for the degree

of

Doctor of Philosophy

in

Neuroscience

MONTANA STATE UNIVERSITY
Bozeman, Montana

November 2005

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November 2005

DEDICATION

I would like to start off by thanking my wonderful thesis advisor Dr. Frances Lefcort. She is my role model as a scientist, wife and mother and showed me that you can balance a research career, family and interests outside of science. I would also like to thank her husband Steve Eiger who makes the best Margarita's outside of Mexico and her two kids Ellie and Isaac for breaks from science that involved make-up time and baseball. I wouldn't have come this far without the "Ladies of the Lefcort Lab", Val my big sister who always knew when it was time for retail therapy and Marta who always brought a different perspective and a much needed hug. Shawn and Judy, fellow graduate students were always nearby to decompress with and Brandon who likes to think he taught me everything I know! I couldn't have done it without the members of the Kulesa Lab whose "odd-topic" conversations are price-less. Danny, Bec, Junior, Cam and Jess thank-you for your quick wit, telling me when I don't have an object in line on the microscope, Wednesday's cookie stealing adventures, happy-hour on the Plaza, Pizza Shapes and gossiping by the coffee pot. Most importantly I need to thank my family. In memory of my grandma, Thelma Maples, who always wanted to know what I was researching, you're an amazing person. My parents, Carolyn and Wayne for being the greatest parents a kid could wish for, who never pushed me but taught me to finish whatever I start. My big sister Angie and younger sister Melanie who are my best friends and are always a phone call away (when the phone is charged that is!) and my big brother Lonnie who I always looked up to and wanted to make him proud of me when I was kid (and as a grown kid!) and always instilled in me to be the best at whatever I did. And of course all my nieces and nephews whose pictures hang by my desk and make me smile everyday I come into work. Maggie I hope you find the joy in school (or atleast in one subject) that I found in science, Max, my baseball buddy, always ready to play catch with me, Morgan my cookie baking and dollhouse buddy, Alexis, whose love and fascination of science I hope keeps growing, Farah my tea party buddy who hasn't decided yet what she likes best (but thinks she likes science), James, aka Jimmy, aka Talent-Ed, I hope you get to do something with trains when you grow up and Landi, the youngest, who I hope can hold her own with all her siblings and be a shining star! Most of all I wish for all my nieces and nephews to find a job they love to get up and go to in the morning as I have found. And last but certainly not least, my husband, Dr. Paul M. Kulesa who had to go through the process of living the graduate student life a gain. I am so glad you were by my side through this, without you I don't know where I'd be. Thanks Buddy! Lastly, I love you ALL!

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ABSTRACT

The neural crest is a unique population of pluripotent cells that are crucial in vertebrate embryogenesis. In the trunk, NCCs migrate along a ventromedial pathway give rise to the dorsal root ganglia or the sympathetic ganglia (SG), or follow a dorsolateral pathway and give rise to melanocytes. Intriguingly, NCCs following the ventromedial pathway migrate in a metameric pattern through the rostral half somite and avoid the caudal somite and this is thought to dictate the metameric pattern of the SG. Static analyses have characterized the development of these structures, but timelapse imaging of NCCs in their normal environment could potentially reveal unidentified cellular and molecular interactions integral to SG development. However, because NCCs migrate deep within the embryo, it is challenging to track NCCs little is known about the cellular mechanisms mediating their migration, aggregation and differentiation.

Here, we follow fluorescently labeled trunk NCCs using a novel sagittal explant culture system and timelapse confocal microscopy. We show trunk NCCs migrate in chain-like formations, and restriction to the rostral somite is not maintained once these cells arrive at the dorsal aorta. Instead, discrete SG only arise after an intermixing of cells along the ventral border of the somite followed by segregation into ganglia. The diverse cell migratory behaviors and active reorganization at the target sites suggest that cell-cell and cell-environment interactions are coordinated with dynamic molecular processes.

In a screen for molecules expressed on NCCs during SG formation, we identified the cell adhesion molecule N-cadherin expressed once NCCs arrive adjacent to the dorsal aorta and form ganglia. Additionally, altering cadherin function drastically alters ganglia size. Additionally, we found EphrinB1 expression was absent adjacent to the dorsal aorta as NCCs had dispersed in this corridor, but strikingly up-regulated in the inter-ganglionic regions after discrete ganglia formation. The ephrinB1 receptor, EphB2, is also expressed on NCCs distributed adjacent to the dorsal aorta when discrete ganglia form. Altering ephrinB1/EphB2 signaling interferes with formation of the primary SG chain and blocks the formation of discrete ganglia. Taken together, these results indicate the importance of adhesive and inhibitory mechanisms in the formation of SG.

CHAPTER 1

INTRODUCTION

Absent in invertebrates, but present in all vertebrates during embryogenesis, neural crest cells (NCCs) comprise a transient population of pluripotent cells that are generated from the dorsal region of the recently formed neural tube along the entire body axis in a rostrocaudal gradient (LeDouarin and Kalcheim, 1999). NCCs born in the head region give rise to cartilage, bone and connective tissue of the face as well as neurons and glia. Cardiac NCCs give rise to portions of the large arteries and septum. Vagal and sacral NCCs give rise to cells of the enteric ganglia. Finally, trunk NCCs form the dorsal root ganglia (DRG), sympathetic ganglia (SG), adrenal medulla and melanocytes (LeDouarin and Teillet, 1974; Bronner-Fraser, 1986; Teillet et al., 1987; LeDouarin and Kalcheim, 1999).

Generation Of Neural Crest Cells

During the process of neurulation in chick embryos, part of the ectoderm thickens to form the neural plate (Conn, 1995) and the edges elevate to form the neural folds. The neural folds fuse at the midline and separate from the ectoderm, giving rise to the neural tube. The rostral most part of the neural tube differentiates into the hindbrain, midbrain and forebrain to form the adult brain, while the remainder of the neural tube gives rise to the spinal cord. NCCs arise

in the dorsal portion of the neural tube and emigrate from the neural tube into the periphery of the embryo. The most widely accepted model of neural crest induction is the neural default model. According to this model, the default fate of ectoderm is to form neural tissue (Knecht and Bronner-Fraser, 2002). Research has shown that cells are induced to become NCCs by interactions between the epidermis and neural plate (Dickinson, et al., 1995). When presumptive neural plate and epidermal tissues were put next to each other, NCCs formed both *in vivo* and *in vitro* (Moury and Jacobson, 1999; Dickinson et al, 1995; Selleck and Bronner-Fraser, 1995; Liem et al., 1995), and both epidermal and neural plate cells gave rise to NCCs (Selleck and Bronner-Fraser, 1995). Non-neural ectoderm and the dorsal neural tube express members of the bone morphogenetic protein (BMP) family, specifically BMP-4 and BMP-7. Studies have suggested that these molecules are responsible for initiating the formation of trunk NCCs (Liem et al., 1995). BMP-4 has the same ability as the epidermis in generating NCCs from the neural plate (Morgan and Sargent, 1997). Therefore, it is proposed that the diffusion of BMP-4 from the neural plate is one factor that induces NCC formation. However, LaBonne and Bronner-Fraser (1998) show that Wnts induce expression of NCC markers in ectoderm explants in conjunction with BMP inhibitors. Thus, BMPs and Wnt signaling are necessary components of neural crest formation.

Before NCCs migrate out from the neural tube, they express N-cadherin and cadherin-6B (Monier-Gavelle and Duband, 1995), cell adhesion molecules

that are involved in cell-cell interactions. When NCCs delaminate from the neural tube, N-cadherin and cadherin-6B disappear, and instead they express cadherin-7 (Nakagawa and Takeichi, 1995). Cadherin -7 has been suggested to be a migration-promoting cadherin while N-cadherin prevents migration (Dufour et al., 1999). NCCs can be induced to leave the neural tube prematurely when antibodies that block N-cadherin activation are used (Bronner-Fraser et al., 1992). When N-cadherin or cadherin-7 are over-expressed in the dorsal neural tube, NCCs fail to delaminate (Nakagawa and Takeichi, 1998). Furthermore, when mouse cell lines were transfected with either cadherin-7 or N-cadherin, migration was prevented while cadherin-7 promoted migration (Dufour et al., 1999).

NCCs delaminate from the dorsal neural tube, undergo an epithelial-to-mesenchymal transition (EMT) and migrate away from the neural tube throughout the developing vertebrate embryo to specified destinations where they settle and differentiate into many cell types (Weston, 1970; LeDouarin, 1982; Bronner-Fraser, 1986). During EMT, NCCs break cadherin mediated cell-cell interactions (Newgreen and Gibbins, 1982; Newgreen and Gooday, 1985; Akiyata and Bronner-Fraser, 1992) and increase cell-extracellular matrix adhesions (Delannet and Duband, 1992; Newgreen and Minichiello, 1995) to facilitate migration. Interactions between migrating NCCs and surrounding extracellular matrix (ECM) molecules are mediated by integrins (Maschoff and Baldwin, 2000), heterodimeric transmembrane glycoproteins. Integrins bind to

ECM molecules and transducer signals, which result in the activation of intracellular signaling pathways. When antibodies were used against the $\beta 1$ -integrin subunit *in vitro*, trunk NCCs failed to attach to fibronectin and laminin (Lallier and Bronner-Fraser, 1993). Specifically, a significant percentage (50-70%) of NCCs failed to migrate out of the neural tube and those that were able to migrate from the neural tube traveled shorter distances when compared to control explants (Kil et al., 1998). *In vivo* and *in vitro* perturbation experiments using antibodies against fibronectin blocked migration of NCCs, confirming an essential role for fibronectin (Boucaut et al., 1984).

Trunk Neural Crest Cells

More specifically, sympathetic ganglia (SG) precursors are the first cells to leave the neural tube in the trunk, and migrate ventrally through the rostral half of the somite to sites adjacent to the dorsal aorta where the primary SG chain will form. Once through the somite, SG precursor cells respond to the local secretion of BMP-4 from the dorsal aorta which promotes their differentiation into sympathetic neurons. BMPs however, are not needed for the migration and formation of SG (Reissmann et al., 1996; Shah et al., 1996; Schneider et al., McPherson et al., 2000).

Trunk NCCs migrate out of the neural tube along two spatially discrete pathways (Loring and Erickson, 1987; Teillet et al., 1987). The first migratory pathway, the ventromedial pathway, leads the NCCs through the rostral half of

each somite, avoiding the caudal half, in a segmented pattern (Keynes and Stern, 1984) giving rise to the DRG and SG. The second migratory wave, referred to as the dorsolateral pathway, begins approximately 24 hours after the ventromedial pathway (Oakley and Tosney, 1991; Erickson et al., 1992). On this route, NCCs migrate out of the neural tube between the ectoderm and the somites to give rise to melanocytes. An important question in developmental biology is to understand molecular signals patterning NCC migration. To investigate signals in the somites acting on NCCs, Bronner-Fraser and Stern (1991) physically rotated the somatic mesoderm in chick embryos so that the polarity of the somites was reversed. If the pathway of the NCCs was unaffected by molecular cues present in the rostral and caudal halves of somites, then one would expect the NCCs to maintain migration through the rostral most regions of the somites (i.e. caudal half after rotation). However, it was found that the migration of NCCs was also reversed when somites were reversed. After rotation, they migrated through the caudal regions of the somite (rostral tissue; Bronner-Fraser and Stern, 1991). Additionally, these researchers rotated a portion of the neural tube 180°. Here, the NCCs still migrated out from the rotated dorsal (now ventral) neural tube and through the rostral half of the somites, demonstrating that somites and their molecular cues play a dominant role in establishing the segmented migration of NCCs. Altering the rostro-caudal composition of the somites by replacing normal somites with multiple rostral or caudal somatic halves also affects the metameric pattern of DRG and SG, as

well as their size (Kalcheim and Teillet, 1989; Goldstein et al., 1990; Goldstein and Kalcheim, 1991). Grafting of multiple rostral half-somites in place of normal segments created unsegmented, larger than normal ganglia.

Tosney (1988) demonstrated that the sclerotome is the area of the somite essential for proper NCC migration and spinal motor axon outgrowth. When the somites were completely removed by aspiration, the DRG formed but were un-segmented (Tosney, 1988). However, when the dermatome and myotome were removed, leaving only the sclerotome regions of the somites, segmentation occurred normally (Tosney, 1987). It is logical that either the rostral half sclerotome attracts the trunk NCCs and/or caudal half sclerotome repels them (Davies et al., 1990). Recently, research has turned to a molecular approach, investigating attractive and repulsive gradients guiding migration of NCCs.

Rostral/Caudal Somite Mechanisms

Trunk NCCs must choose, upon emigration from the neural tube, between the ventromedial pathway to the DRG and SG or the dorsolateral pathway that incipient melanocytes follow. Early migrating cells following the ventromedial pathway have recently been shown to express the receptors Robo1 and Robo2, while the ligand Slit2 is expressed in the dermamyotome. *In vitro*, Slit2 repels migrating NCCs and a dominant negative form of the Robo1 receptor induces precocious migration of NCCs ectopically into the dorsolateral pathway.

Additionally, ephrinB1 is expressed in the dermamyotome and inhibits NCCs expressing the EphB2 receptor from entering the dorsolateral pathway. Later, however, it promotes the migration of migrating EphB2 expressing cells along the dorsolateral pathway (Santiago and Erickson, 2002). A change in the response of melanoblasts as compared to ventromedially migrating cells is due to ephrin-B proteins enhancing melanoblast adhesion to fibronectin, promoting migration. Oakley et al. (1994) also found that high levels of versican-like glycoconjugates correlates with absence of migration, while a decrease in the immunoreactivity for these compounds coincides with melanoblast migration.

In addition to blocking the dorsolateral pathway for early ventromedially migrating cells, the Eph/ephrin family of receptor tyrosine kinases also helps pattern NCC migration through the somites. Wang and Anderson (1997) used *in vitro* assays of rat neural tubes to show that both ephrinB1 and ephrinB2 repel migrating NCCs. They demonstrated that ephrinB1 is expressed in the caudal half of the sclerotome at the appropriate time in rat embryonic development to cause inhibitory effects. Krull et al. (1997) examined ephrinB1 and EphB3 with respect to chick NCCs and found that EphB3 and EphB2 are expressed in the rostral half sclerotome as well as on NCCs, while ephrinB1 is expressed in the caudal half sclerotome. Furthermore, by adding soluble ephrinB1-Fc to the somites, which blocks normal receptor-ligand interactions, a loss of the normal migratory pattern of NCCs was observed (Krull et al., 1997). Stripe assays also performed by Krull et al (1997) using chick neural tubes confirmed that NCCs

avoid specific ephrins. Recently, McLennan and Krull (2002) showed ephrin-As and EphA4 promote trunk NCC migration, as NCCs express EphA4 and in culture migrate on ephrin-A substrates. These results suggest that migration through the rostral half sclerotome is not a default pathway, but one that includes positive and negative guidance cues.

Sema3A, a soluble molecule which belongs to the semaphorin family of proteins, also influences NCC migration. Expressed in the dermamyotome and ectoderm regions during early development (Shepherd et al., 1996), as well as in the caudal somite (Eickholt et al., 1999), Sema3A was first shown to inhibit the extension of axonal growth cones *in vitro* (Shepherd et al., 1996). More recently, Eickholt et al. (1999) showed that trunk and cranial NCCs avoid Sema3A rich regions. When given the choice of stripes composed of fibronectin or fibronectin plus Sema3A, trunk and cranial NCCs preferred fibronectin only stripes (Eickholt et al., 1999), suggesting that Sema3A is involved in proper segmental migration of trunk NCCs. Lectin peanut agglutinin-binding glycoproteins are present in the caudal half of the sclerotome (Stern et al., 1986; Davies et al., 1990) avoided by migrating NCCs. Explants treated with lectin peanut agglutinin-binding glycoproteins showed abnormal NCC migration (Krull et al., 1995). Specifically, NCCs migrated into both the rostral and caudal halves of somites instead of migrating through only the rostral half (Krull et al., 1995). Another molecule, F-spondin, an ECM molecule, is expressed in the somatic regions that NCCs avoid, namely the dermamyotome and the caudal half sclerotome. F-spondin

containing media inhibited the migration of NCCs from the neural tube in trunk explant assays (Debby-Brafman et al., 1999). Furthermore, when F-spondin antibodies were injected into chick embryos before trunk NCCs had migrated, NCCs subsequently migrated into areas normally avoided, including the caudal half sclerotome and dermamyotome. Over-expression of F-spondin inhibits the emigration of NCCs from the neural tube (Debby-Brafman et al., 1999).

In addition to substratum cues guiding NCC migration, soluble proteins can also influence the trajectory of NCCs. The neuregulins, EGF-like growth and differentiation factors signal through receptor tyrosine kinase receptors of the ErbB family. ErbB2 is expressed on migrating NCCs and mouse knock-outs of ErbB2, ErbB3 or neuregulin-1 genes cause hypoplasia of the primary SG chain (Britsch et al., 1998). Neuregulin-1 is expressed along the pathway of NCCs to the target site of the dorsal aorta, and in mutant mice, although NCCs migrate from the neural tube, they accumulate at dorsal locations near the DRG. Therefore, NCCs are unable to migrate ventrally to populate the SG.

Stop Signals For Neural Crest Cells

NCCs migrating along the ventromedial pathway must choose to either stop adjacent to the neural tube and populate the DRG, continue ventrally towards the dorsal aorta to populate the SG or continue on to form the enteric nervous system. Exactly how the diverse fates of these cells are determined and how their specific migration is directed are key questions in developmental

biology. Specifically, the stop signals that regulate the cessation of migration at sites of DRG and SG and their formation are only partially understood.

Recently, the chemokine stromal cell-derived factor-1 (SDF1) was shown to attract sensory neuron progenitors (Belmadani et al., 2005). Chemokines are small secreted proteins that exert their effects by activating G-protein coupled receptors and play several fundamental roles in leukocyte development and migration (Tran and Miller, 2003). Chemokines also play important roles in central nervous system development (Tran and Miller, 2003). Belmadani et al (2005) showed that migrating NCCs and NCCs in the DRG express the chemokine receptor CXCR4. The ligand for CXCR4, SDF1, is expressed along the pathway taken by NCCs to the DRG and CXCR4 mutant mice had small and malformed DRGs. Belmandi et al (2005) also showed *in vitro*, that SDF1 acted as a chemoattractant for NCCs. NCCs expressing CXCR4 interestingly also generally stained for Brn3a, a transcription factor for DRG neurons, whereas only a small number of CXCR4 positive cells expressed the transcription factor Phox2b, indicating catecholaminergic phenotype of sympathetic neurons. These results indicate the ability of the chemokine SDF1 to segregate the progenitors of the DRG from the SG. Thus, this family of molecules influences cell migration in more than one cell type. Potentially, another chemokine receptor could sort and signal SG ventrally toward the dorsal aorta.

Neural Crest Cell Lineage Analysis

NCCs comprise a unique set of embryonic cells with the developmental potential to produce many diverse cell types including neurons, glia, bone, cartilage and dermis. Early NCCs migrate and become widely dispersed in the developing embryo and along the way respond to local environmental cues to ultimately produce cell types appropriate for their terminal locations. *In vivo* lineage tracing experiments revealed premigratory NCCs are multipotent (Bronner-Fraser and Fraser, 1989; Frank and Sanes, 1991). Other single cell progeny studies carried out on trunk NCCs suggests the existence of cells with various degrees of restriction: tripotent, bipotent and monopotent (reviewed by Sieber-Blum, 1990; Bronner-Fraser and Fraser, 1991). In a study by Dupin and LeDouarin (1995), it was found that about 80% of clones from labeled single cells were derived from multipotent progenitor cells with a progeny composed at least of 2-4 different cell types in various combinations. Taken together, these results indicate that although a majority of progenitors within the neural crest are pluripotent, a considerable portion of cells derive from early-specified precursors.

Interestingly, Henion and Weston (1997) compared NCC potential immediately after leaving the neural tube and at different time intervals prior to differentiation. Early migrating NCCs analyzed after 6 hours in culture showed roughly 50% were fate restricted, whereas at 36 hours after explantation, 77% were fate restricted. Additionally, Luo et al., (2003) demonstrated that distinct neurogenic and melanogenic sublineages diverge before or soon after NCCs

emerge from the neural tube, and can be distinguished by cell type-specific expression of receptor tyrosine kinases. Lo et al. (2005) described a late-emigrating NCC population that is strongly biased toward a sensory but not autonomic fate, and implicate locally synthesized GDF7 in this fate restriction. These results show that segregation of cell lineage is a progressive and non-random process. Anderson (1989) very elegantly compared NCC lineage diversification to the hemopoietic system. The initial neural crest progenitor cell is similar to the multipotent hemopoietic stem cell that gives rise to several terminally differentiated derivatives by ultimately generating a series of committed progenitor cells, which are restricted to distinct sublineages. Many neural crest derived cells do not undergo differentiation until after they disperse. Consequently, it has been unclear whether the appearance of differentiated cells in precise locations is the result of the differential localization of developmentally undefined precursors to adopt specific fates. A major unresolved question is when the fates of individual NCCs become different from one another.

Several transcription factors have been identified that specify sensory and autonomic cell fate by isolating vertebrate homologues to invertebrate neuronal determinants in *Drosophila* (reviewed by Ghysen and Dambly-Chaudiere, 1988). Identifying and isolating vertebrate homologous genes involved in determining the sympathoadrenal lineage led to the cloning of the rat achaete-scute homologue MASH-1 (CASH-1 in chick; Johnson et al., 1990; Ernsberger et al., 1995). Expression of Mash-1 occurs in NCCs just after they colonize in the SG

anlagen, preceding other markers, and not detected in sensory ganglia, suggesting that this transcription factor is a specific marker for autonomic progenitors of the peripheral nervous system (Lo et al., 1991; Guillemot and Joyner, 1993). This was followed by the isolation of neurogenin transcription factors which are expressed early in sensory but not autonomic lineages (Ma et al., 1996). Understanding how and when these transcription factors are regulated will begin to tell us when lineage restriction specifically occurs for different cell types.

Functions Of The Autonomic Nervous System

The autonomic nervous system (ANS) derives entirely from NCCs and includes SG, parasympathetic ganglia, adrenal chromaffin cells, and the enteric ganglia. The sympathetic nervous system regulates blood pressure, vasal dilation and control and innervation of smooth muscle, cardiac muscle and secretory glands. Activation of the sympathetic system in response to stress or emergency prepares the body for fight or flight response. The efferent division of the sympathetic part of the ANS is organized as a two-neuron pathway from the central nervous system to the peripheral organs. In the SG, the information is transmitted from the pre- to the postganglionic neurons, and the ganglia are characterized as paravertebral, prevertebral, previsceral or terminal by their anatomical location. The paravertebral ganglia are located on both sides of the vertebrate column, forming the two sympathetic chains. The main transmitter of

the peripheral sympathetic nervous system is noradrenaline, and the majority of the ganglion cells are noradrenergic (Kirby and Gilmore, 1976; Allen and Newgreen, 1977). There are hereditary sensory and autonomic neuropathies, one of which is familial dysautonomia where NCC development doesn't happen normally. Loss of normal SG results in inability to control body temperature and blood pressure, vital involuntary actions of the body.

Secondary SG Chain Formation

The primary SG chain forms on either side of the dorsal aorta. However this is not the permanent structure of the SG. Their final position lies medial to the branch point of the intercostals nerve and the prevertebral projections, immediately ventral to the DRG (Cornbrooks et al., 1997). The permanent secondary sympathetic chain forms from a dynamic multi-step process involving two distinct migratory steps (Tello, 1925; Kirby and Gilmore, 1976). NCCs that follow the ventromedial pathway from the neural tube stop immediately adjacent to the neural tube, coalesce and form the dorsal root ganglia or continue ventrally through the length of the somite towards the dorsolateral aspect of the dorsal aorta and contribute to the primary sympathetic chain (Thiery et al., 1982; Teillet et al., 1987; Loring and Erickson, 1987; Lallier and Bronner-Fraser, 1988). Permissive and inhibitory cues in the environment and ECM contribute to NCCs arriving at the dorsal aorta. Newgreen et al. (1985) showed that the ECM around the notochord is an inhibitory zone to NCCs. Pettway et al. (1990) specifically

showed chondroitin-containing proteoglycans to be responsible for the inhibitory response by NCCs. Debby-Brafman et al. (1999) showed that the paranotochordal sclerotome synthesizes F-spondin and microinjecting a neutralizing antibody allowed NCCs to migrate adjacent to the notochord. The permanent secondary sympathetic chain arises from a subsequent dorsolateral migration of cells from the primary chain 24 hours after forming ganglia adjacent to the dorsal aorta (Tello, 1925; Kirby and Gilmore, 1976). Very little is known about the process, cell behavior and dynamics that occur, most likely due to the location of the migratory path from the primary chain to the secondary chain deep within the embryo.

Using Imaging To Understand Neural Crest Cell Behavior

Given that a common pool of progenitor cells gives rise to the DRG and SG, which migrate ventromedially along the same path within the embryo, understanding their migratory behavior as they differentiate and segregate into these two structures and how they decide to stop in the correct location to form ganglia are important questions in developmental biology. Imaging trunk NCCs in their native environment could potential identify unknown cell-cell and cell-environment interactions that mediate these events that are not apparent from static images alone.

Due to their dorsal-to-ventral migration deep within the embryo, the precursor cells of the SG have been difficult to analyze. To solve this problem,

we developed a trunk sagittal explant technique that has provided a new way to investigate cell-cell and cell-environment interactions that mediate SG formation. By gaining access to this restricted area in whole embryo and trunk explants, we can begin to understand the cellular behavior of SG precursors from their earliest stage of generation to their permanent secondary sympathetic chain location. Using our novel explant technique with timelapse confocal microscopy, we were able to follow NCCs as they migrate from the dorsal neural tube and stop adjacent to the neural tube to form the DRG and also those that continue ventrally to the dorsal aorta. Upon imaging these cells in their native environment as they formed ganglia, we found three striking behavior of these cells not observed before. First, NCCs migrate in chains through the rostral somite. Secondly, once NCCs migrate past the ventral edge of the sclerotome to the dorsolateral aspects of the dorsal aorta, cells no longer maintain their segregated patterns as they did through the somites, but rather distribute in the anterior and posterior direction to become continuous adjacent to the dorsal aorta. Lastly, the second segregation process to form discrete ganglia is a highly dynamic process with constant cell rearrangement and extensive filopodial contacts.

To understand the molecular mechanisms mediating the novel cellular behaviors revealed using timelapse imaging, we performed an extensive expression screen using various antibodies against molecules shown to influence cell migration. We examined expression patterns at Hamburger and Hamilton

(HH; 1951) st.17, when NCCs have dispersed adjacent to the dorsal aorta and HH st.20, when discrete ganglia have formed. Interestingly, Eph/ephrins and N-cadherin were shown to be expressed in the correct spatio-temporal pattern to potentially influence ganglion formation.

EphB2 is expressed by migrating NCCs and has been shown to interact with ephrinB1 in the caudal sclerotome to inhibit the NCCs from entering this area (Krull et al, 1997). Interestingly, EphB2 is expressed on NCCs throughout migration and formation of SG and ephrinB1 is upregulated in the inter-ganglionic region between SG at HH st.20. Blocking this inhibitory interaction locally using Fc-fusion proteins disrupted ganglion formation and cells remained continuously distributed adjacent to the dorsal aorta.

Additionally, N-cadherin is expressed on pre-migratory NCCs in the dorsal neural tube and down-regulated when they begin migration (Theiry et al., 1982a; Duband and Theiry, 1987; Monier-Gavelle and Duband, 1995). It had been shown that cells re-express N-Cadherin in the DRG and SG (Theiry et al., 1982b; Duband et al., 1985). We find that N-Cadherin is upregulated on NCCs at HH st.17 and persists through HH st.20 in agreement with Monier-Gavelle and Duband (1995). Using N-cadherin blocking antibodies and mis-expression constructs we show that discrete ganglia are generated but their size and area are dramatically altered.

Taken together, these results demonstrate that inhibitory and attractive mechanisms work together to sculpt the formation of the primary SG chain.

Surprisingly, we have only characterized half of the migration and development of the SG. Roughly 24 hours after forming the primary SG chain, these cells undergo a dorsal-ward migration to their final position ventral to the DRG and ventral root. Using cultured transverse sections we are just beginning to analyze the migratory behaviors of these cells during this second migratory phase. Much like their ventral-ward migration, these cells are highly dynamic with numerous filopodial contacts extending in all directions from the cell and contacting many neighbors at the same time.

An important question in developmental biology and also the focus of this thesis is to understand how precise spatial patterns of cell types are maintained despite rearrangements of cells both during and after migration to their target location. Using timelapse confocal microscopy combined with molecular biology techniques, we were able to conduct a detailed spatial-temporal analysis of key events involved in the formation of SG, a major component of the peripheral nervous system.

CHAPTER 2

NOVEL SAGITTAL EXPLANT CULTURE TECHNIQUE

Introduction

This chapter describes a new culture and imaging technique to follow individual, fluorescently labeled neural crest cells in sagittal slice explants of the chick neural tube, trunk region, with video and confocal time-lapse microscopy.

Area Of Application

This technique has been used to study chick neurogenesis. Namely, the patterning and cell migratory behaviors of neural crest and neural progenitor cells that form the dorsal root ganglia (DRG) and sympathetic ganglia (SG). We have followed fluorescently labeled cells in time-lapse microscopy for on average 24 hours and up to 36 hours. We have imaged embryos as young as 45 and up to 84 hours of incubation (approximately HH st.16-24). This technique may be used in both younger and older embryos, for example, to study somite formation and limb development.

Materials

1. Fertile White Leghorn chick eggs incubated at 38°C in an egg incubator (VWR, WL51475).
2. Forceps and Scissors (Fine Science Tools; 11252-30 & 14060-10)

3. Needles (25 & 27 gauge, Becton Dickinson) and syringes (VWR, 1 & 5 ml)
4. Petri dishes (Becton Dickinson, 35x10mm and 100x15mm)
5. Millicell culture plate inserts (Millipore, PICMORG50)
6. Glass coverslips, 25mm circles (Fisher Scientific, 12-545-102)
7. Sandpaper; fine, general purpose
8. Fibronectin, 20 μ g/ml in phosphate buffer (Gibco, 33016-015)
9. Tungsten needles (A-M Systems, 717000)
10. Neural basal medium (Gibco, 21103-049)
11. B27 supplement; serum free (Gibco, 17504-044)
12. High vacuum grease (Dow Corning, 59344-05)
13. Plastic transfer pipets (Samco, 202-205)
14. Soldering iron, general use model
15. We use a laser scanning confocal microscope (Zeiss LSM Pascal) on an inverted fluorescent compound microscope (Zeiss Axiovert)
16. Coin (preferably the size of a U.S. dime, 18 mm diameter)
17. Razor blades
18. Filter paper (Whatman, 1001185)
19. Heater box for microscope (A cardboard box with sides large enough to fit around the microscope stage)
20. Foil Insulation (Reflectix, BP24025)
21. Table top incubator (Lyon Electric, 950-107)

22. Digital thermometer (Fisher, 15-077-17A)

Protocol and Procedures

Embryo Preparation

1. Rinse eggs with 70% alcohol and remove 3 ml of albumin from the caudal part of the egg with the 5 ml syringe and 27 needle.
2. Perform any cell and tissue labeling or embryo manipulations prior to generating sagittal explants.
3. Cut a hole in the eggshell large enough to manipulate around the embryo, discard shell.
4. Place a few drops of Ringer's solution around the embryo.
5. Cut a circle with a hole in the middle out of filter paper and place over the egg so the embryo is in the middle of the hole.
6. Using scissors, cut a circle around the outer perimeter of the circle through the surrounding membranes and blood supply.
7. With a pair of tweezers gently remove embryo and transfer to a 100mm petri dish with Ringer's solution.

Sagittal Slice Preparation

8. Using a tungsten needle, cut out an explant region of the embryo in the region from which you wish to cut the sagittal slice and transfer to a new petri dish with fresh Ringer's solution (Fig. 1a).
9. Lay explant dorsal side up.

10. Position explant between forceps to hold in place.
11. Using a tungsten needle, lightly cut along the midline of the spinal column down the entire length of the explant (cutting through the ectoderm is sufficient to allow the spinal cord to begin to splay open) (Fig. 1b).
12. While holding the embryo in place with the forceps, in one motion, insert the razor blade in the incision made with the tungsten needle, and slice down through the rest of the embryo. This incision will slice the embryo in half and produce two sagittal explants.

Millicell Culture Insert And Petri Dish Assembly

13. Remove Millipore filter from package, place in large petri dish and coat surface with fibronectin ($\cong 100$ ul). Incubate in covered petri dish for 15 minutes.
14. Remove excess fibronectin by tilting filter and pipeting excess from the wall of the filter.
15. Coat the membrane with Ringer's solution and let it stand until ready.
16. To prepare the petri dish, place a dime in the center of the bottom of a 10x15mm petri dish.
17. Heat the dime with a soldering iron, applying gentle pressure with the iron on the dime. When sufficiently heated, the dime will melt the bottom of the petri dish, and push through it leaving a hole in the dish.

18. Cut away excess plastic around hole and use sandpaper to smooth the perimeter of the hole.
19. Clean the dish prior to use with ethanol and wipe clean with a kimwipe.
20. With a 1ml syringe, apply a thin layer of vacuum grease around the hole on the inside of the petri dish.
21. Clean a 25mm coverslip with a kimwipe and ethanol.
22. Place coverslip on top of the hole and gently seal it against the vacuum grease with the blunt end of a pair of forceps.

Microscope Stage Heater Box Assembly

23. With a cardboard box large enough to fit around the stage and base of the microscope, cut the box to conform and fit snugly around the microscope.
24. On the outside of each side of the box, attach the thermal insulation with clear packing tape.
25. Depending on the size/layout of the microscope, decide which side of the box to cut a hole into large enough to accommodate the diameter of the incubator heater.
26. Feed the heater through the inside of the hole and pull out till the box catches on the outer lip of the incubator to hold it in place.
27. Make sure to cut holes in various locations in order to manipulate the microscope controls and have access to the stage.

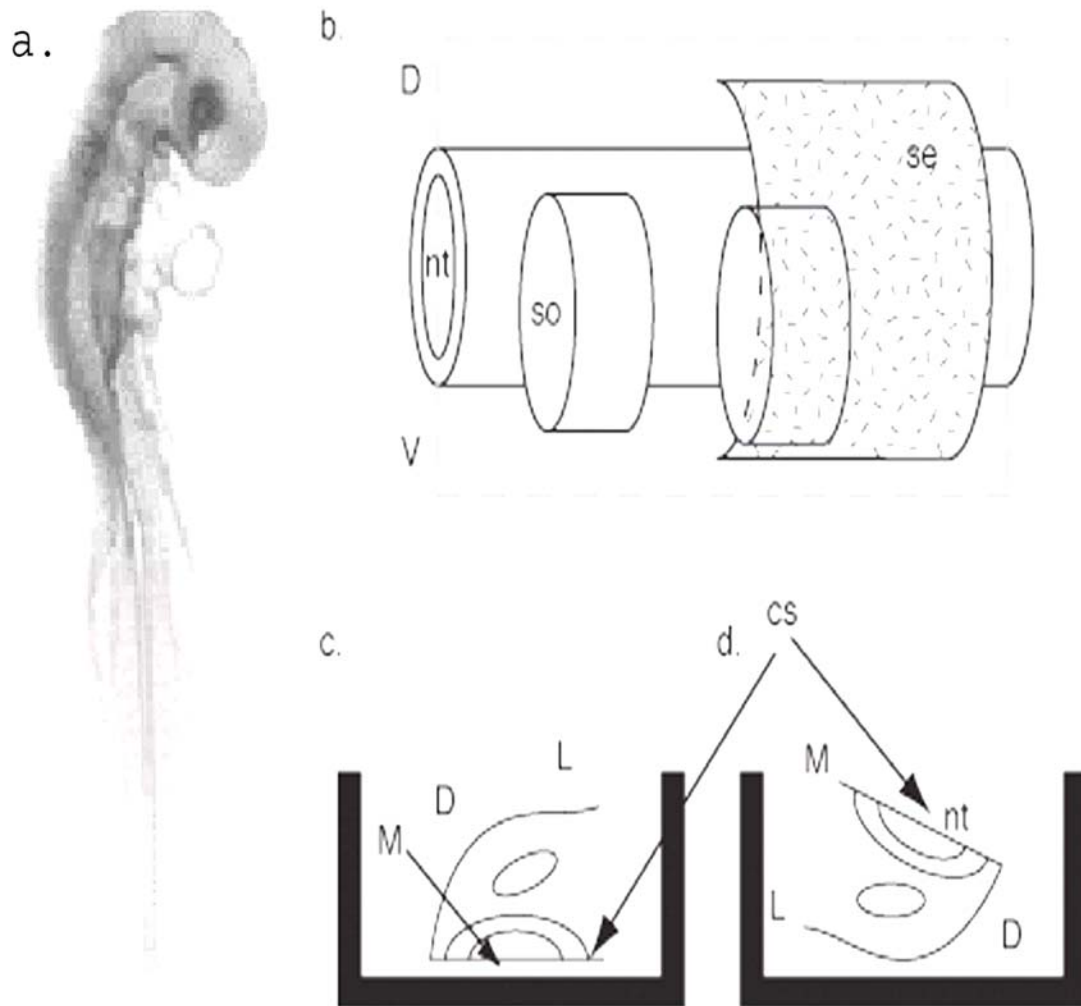


Figure 1. Schematic of sagittal explant technique. A. 72 hour chick embryo, trunk explant taken from highlighted region. b. Sagittal slice of trunk explant. Dotted lines indicate plane of cut. C. Lateral surface up explant. D. Lateral surface down explant. D=dorsal side of embryo, V=ventral side of embryo, L=lateral surface of explant, M=medial surface of explant, nt=neural tube, so=somite, se=surface ectoderm.

28. Cut a hole in the top of the box to load your sample onto the scope through, and for the top of the scope to sit down through.

Culture Preparation

29. Prepare neural basal media by adding B27 supplement at 1:50.
30. Place 1ml of B27 supplemented neural basal media into bottom of petri dish.
31. Place Millipore filter on top of coverslip inside of petri dish.
32. Rinse explant a few times in fresh Ringer's solution by carefully pipeting up and down.
33. Gently transfer the explant to the Millipore filter.
34. Use forceps to orient the explant medial or lateral side down, depending on area that is to be visualized.
35. Pipet excess liquid off of the Millipore filter and from inside the petri dish so the filter is sitting against the coverslip.
36. Place the lid on petri dish and seal with a piece of parafilm.

Microscope Preparation

37. Place box and incubator around the microscope and set incubator temperature to 38°C.
38. Place the probe of the digital thermometer next to the microscope stage such that it is an accurate readout of the temperature near the stage.

Discussion

We utilized the sagittal slice explant technique to study neural crest cell migration and genesis of the chick peripheral nervous system (PNS). Sagittal slice explants were taken from 72-hour (HH st.20) chick embryos that had been injected with a GFP encoding vector into the neural tube at HH st.10 (Fig. 1a). Sagittal slice explants were prepared by cutting the tissue along the vertebrate axis (Fig. 1b), and laid either lateral side down (Fig. 1c.) or up (Fig. 1d.) on a filter. The sagittal explant is taken from the highlighted area (Fig. 1a.).

To investigate the formation of the sympathetic ganglia (SG; Fig. 1.c,) fluorescently labeled trunk neural crest cells were visualized migrating out of the neural tube and coalescing to form the SG using time-lapse confocal microscopy. Images taken from a typical time-lapse imaging session show the time evolution of the SG (Fig. 2). Neural crest cells in the more anterior region of the explant emerge and migrate through the rostral half of the somites (Fig. 2.b-d) and stop in the periphery to form the SG (Fig. 2.e-g).

Advantages And Limits

The sagittal slice culture technique allows the visualization of exposed interior (medial) surfaces from a sagittal perspective. With the cut made down the midline of the embryo, events can be imaged which occur along the anteroposterior and dorsoventral axes of the embryo. With this technique, much

of the normal embryo morphology is maintained since more of the intact embryo is retained in the explant. Previously, it had been difficult to study morphogenetic events that occur deep within the embryo.

The sagittal slice explant technique allows for visualization of morphogenesis in later stage embryos and in embryos where cell migratory paths occur in the dorsoventral plane. Imaging cellular events at later embryonic stages has been technically difficult due to complexities in embryonic configuration (i.e. flexures and twists) and because of movements associated with the beating heart. This technique obviates some of these problems by allowing tracking of fluorescently labeled cells that migrate along the surface of the exposed medial structures and those into deeper tissue because the heart is separated from the slice explant. In addition, it has been difficult to follow cell trajectories along dorsoventral paths in typical cross-section slices. However, the sagittal slice technique allows for these cell migratory pathways to be visualized. Hence, our sagittal slice explant method has proven ideal for imaging morphogenetic events which occur in later stage chick embryos in the dorsoventral plane, such as formation of the DRG and SG.

With this preparation, two imaging approaches are possible: 1) cells that migrate and remain superficially along the dorsal-lateral surface of the explant can be imaged and/or 2) the medial surface of the explant can be imaged to track cells that migrate within or adjacent to the spinal cord. Furthermore, because of the rostral-caudal temporal developmental gradient, one can image migrating

cells at sequential stages of development within a single explant, depending upon its length and the extent of cell labeling along the rostral-caudal axis.

The limitations to the sagittal slice culture technique include typical difficulties associated with slice culture preparations. First, there is a loss of tissue morphology over time. Second, outside of the egg, the embryo is subjected to a decrease in nutrition which induces loss of structure and degradation after 40 hours of incubation. Third, a sliced explant of an embryo has regions of its internal tissue exposed to the external environment. These excisions will induce a wound healing affect on the neighboring tissue and slightly alter the morphology as compared to an *in vivo* embryo. However, the last aspect can be partially corrected by placing the cut slightly lateral to the midline of the neural tube to include the entire neural tube.

The chick sagittal slice technique has several advantages over other slice culture or whole embryo techniques. These include the access to morphogenetic events within a three dimensional embryo at early and late stages (HH st.13-26) of development and an opportunity to visualize phenomena along the vertebrate anterior-posterior axis. This is important since many critical patterning events occur in a rostral to caudal manner in the embryo. Thus, the sagittal slice culture offers an ability to study neural development in living tissue and provides a viable option for imaging events which occur in the dorsal-ventral plane of the embryo and thereby obviate difficulties of imaging deep within whole embryos.

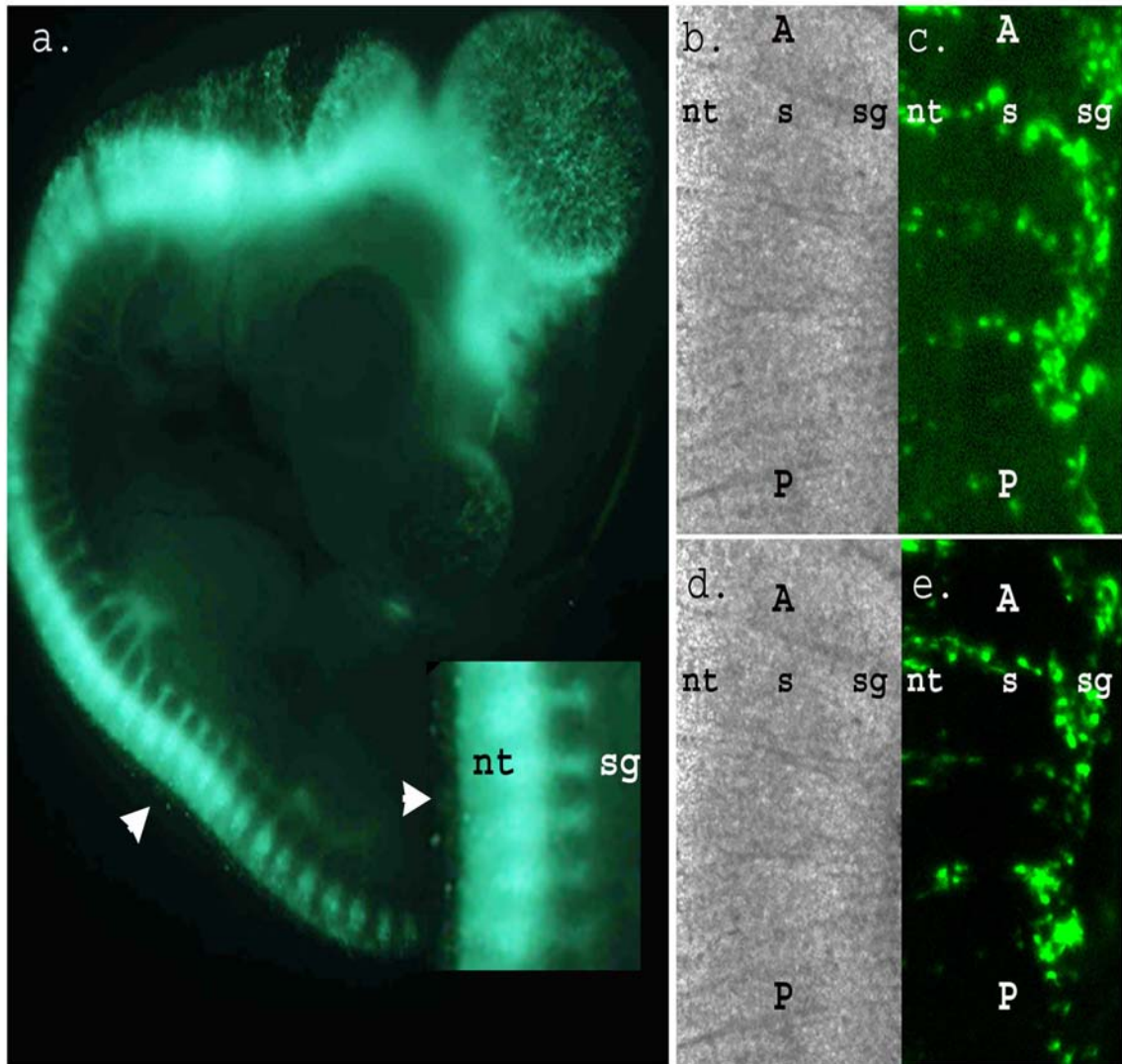


Figure 2. Before and after images taken from a typical time-lapse imaging session using the sagittal explant technique. A. 84 hour chick embryo fluorescently labeled at t=36 hours. Sagittal explant taken from highlighted trunk region. B-D. Lateral side down image at t=0, brightfield, fluorescence. E-G. Lateral side down image at t=15 hours, brightfield, fluorescence. A=anterior, P=posterior, nt=neural tube, so=somite, sg=sympathetic ganglia.

CHAPTER 3

IMAGING NEURAL CREST CELL DYNAMICS DURING FORMATION
OF DORSAL ROOT GANGLIA AND SYMPATHETIC GANGLIAIntroduction

The neural crest is a highly migratory population of cells that gives rise to many diverse cell derivatives within the body. Neural crest cells (NCCs) emerge from the dorsal region of the fusing neural tube, migrate along stereotypical pathways through the embryo and differentiate into the vast majority of the cell types and tissues that comprise the facial skeleton, aspects of the cardiovascular system, peripheral nervous system (PNS), and sympathoadrenal system (Weston, 1970; LeDouarin, 1982; Bronner-Fraser, 1986). NCCs arise at all axial levels of the embryo, and emigrate in a temporal gradient anterior to posterior, with migration initiated in the cranial region. Trunk NCCs give rise to melanocytes, glia and neurons of the dorsal root ganglia (DRG) and autonomic ganglia including sympathetic ganglia (SG) and parasympathetic ganglia (Weston 1963; LeDouarin 1982; Teillet et al., 1987; Lallier and Bronner-Fraser, 1988), which form in a metameric pattern along the anterior-posterior (AP) axis of the embryo. Although static studies have elegantly characterized the lineage of individual trunk NCCs (Fraser and Bronner-Fraser, 1991; Frank and Sanes, 1991; Zirlinger et al., 2002), timelapse imaging of live, motile NCCs navigating through their normal environment could potentially identify unknown cellular and

molecular interactions not apparent by analysis of static images (Lichtman and Fraser, 2002). However, because trunk NCCs migrate ventrally deep within the embryo, very little is known about the cellular mechanisms that mediate their migration, aggregation and differentiation as they form the metameric array of DRG and SG.

The routes navigated by migrating trunk NCCs have been well characterized: those NCCs that take a ventromedial route migrate through the somatic mesoderm, stop midway along and lateral to the neural tube, coalesce and give rise to the DRG. A subpopulation of trunk NCCs that continue ventrally past the formation site of DRG, accumulate dorsolateral to the dorsal aorta and give rise to the SG. Neural crest cells that migrate dorsolaterally underneath the ectoderm become melanocytes (Tosney, 1978; Erickson et al., 1980; Theiry et al., 1982; Loring and Erickson, 1987). Neural crest cell migration through the somites is patterned: cells enter the rostral half but avoid the caudal half of the somite (Keynes and Stern, 1984; Rickman et al., 1985; Bronner-Fraser, 1986) and this stereotypy generates segregated streams of cells which form the basis for the metameric pattern of the DRG, SG and spinal motor axons (Bronner-Fraser, 1986; Lallier and Bronner-Fraser, 1988; Oakley and Tosney, 1993). Experimental evidence implicates extrinsic environmental cues localized in the somites in guiding trunk NCCs. When presumptive somites are rotated by 180°, NCCs and motor axons migrate through the (now) caudal somite (Bronner-Fraser and Stern, 1991). This segregation of NCCs to follow a specific migratory route

or corridor suggests that the function of the operative molecular mechanisms is to generate segregated lateral structures, such as the DRG and SG.

Studies investigating the expression patterns of candidate molecules influencing the migratory patterns of NCCs in vitro (Erickson and Perris, 1993) and in vivo (Bronner-Fraser, 1986) have implicated neuregulins, BMPs, semaphorins/collapsins and the Eph/ephrins family of molecules (Krull, 2001; Graham, 2003). Upon entering the sclerotome, Eph-family receptors and their ephrin ligands mediate repulsive interactions between NCCs and caudal half-sclerotome cells, thereby restricting neural precursors to specific territories in the developing nervous system (Krull et al., 1997; Wang and Anderson, 1997). In addition to avoiding the actively inhibitory cues present in the caudal half of each somite, there is evidence for the presence of attractive, positive molecular interactions that influence NCC migration through the rostral half of each somite (Koblar et al, 2000; Krull, 2001). The stop signals that regulate the cessation of migration at sites of DRG formation are less characterized, however, an intact β -catenin signaling pathway within NCCs is required for the formation of DRG anlagen (Hari et al., 2002) and DRG often form in aberrant locations in the absence of sonic hedgehog signaling (Fedtsova et al., 2003). Sympathetic precursors respond to the local secretion of BMP-4 from the dorsal aorta, which is necessary for inducing differentiation of mature sympathetic ganglia, although its exact role in the migration of NCCs has not been elucidated (Reissmann et al, 1996; Shah et al., 1996; Schneider et al., 1999; McPherson et al., 2000).

Targeted deletion of neuregulin, erbB2 or erbB3 genes all result in a marked hypoplasia of the primary chain of SG (Britsch et al., 1998). In these mutants, NCCs emigrate normally from the neural tube but fail to migrate ventrally towards the dorsal aorta and hence to contribute to SG anlagen formation; instead their migration is arrested dorsally in the vicinity of the DRG anlagen. Thus, these data implicate neuregulin and its receptors in the ventral migration of NCCs, although the mechanisms by which they effect migration remain incompletely characterized. Another guidance molecule, Sema3A and its receptor neuropilin-1 are required for the arrest and aggregation of sympathetic neural precursors at their normal site dorsolateral to the dorsal aorta (Kawasaki et al., 2002; Bron et al., 2004) although the cellular mechanisms mediating these steps have not been elucidated. Interestingly, it has been shown that trunk NCCs do not respond to guidance cues known to influence the cranial NCCs (Bronner-Fraser, 1993), indicating differences in either the environmental guidance cues present at the two levels and/or in the guidance receptors expressed by cranial vs. trunk NCCs.

Due to their dorsal to ventral migration to deep within the embryo, the cell-cell and cell-environment interactions that mediate DRG and SG formation have been difficult to analyze in intact developing embryos. Cell tracking studies of fluorescently-labeled cranial NCCs revealed a rich set of cell migratory behaviors, including collective chain-like cell arrangements suggesting that a sophisticated set of underlying patterning mechanisms and extrinsic cues play a role in sculpting the migration pattern (Kulesa and Fraser, 1998; Teddy and

Kulesa, 2004). In contrast, there is a paucity of knowledge on the migratory behaviors of NCCs in the trunk region. A very useful trunk explant technique designed to image the early sorting of trunk NCCs into streams (Krull et al., 1995) is insufficient for imaging later events due to complications with tissue thickness and the beating of the developing heart. Several groups have used transverse slice explants to study early events in the development of the PNS (Hotary et al., 1996; Krull and Kulesa, 1998). However, this system is not ideal for imaging structures that develop along the vertebrate rostrocaudal axis, such as the DRG and SG.

In this study, we investigate the cellular dynamics that mediate DRG and SG formation. We follow fluorescently labeled trunk NCCs using a novel sagittal explant technique and time-lapse confocal microscopy (Kasemeier et al., 2004). To our knowledge, this is the first study in which DRG and SG formation has been described in spatio-temporal detail. We show that along their dorsoventral migratory route, trunk NCCs are highly motile and dynamically interact with neighboring cells and the environment via an elaborate extension and retraction of filopodia. Some cells migrate collectively, forming chain-like arrangements that stretch from the DRG to the SG. Surprisingly, the segregated pattern of NCC streams is not maintained once cells arrive at the presumptive SG sites. Instead, cells disperse and intermix along the AP axis and contact cells in the neighboring SG sites. Here we document this segregation process in detail and reveal for the first time the highly dynamic filopodial activity that transforms an initially

continuous stream of cells into discrete, segregated SG. By imaging crest cells we also found that rerouting of NCCs between developing DRG and SG is temporally regulated in that early migrating cells, but not later migrating cells, can reverse their direction of migration once they have arrived in their target ganglion (DRG vs. sympathetic). The diverse cell migratory behaviors and active reorganization at the target sites suggest that cell-cell and cell-environment interactions are coordinated with dynamic molecular processes to ultimately sculpt the organization of the peripheral nervous system.

Materials And Methods

Embryos

Fertilized White Leghorn chicken eggs (Spafas Avian products; North Franklin, CT) were placed in a rocking incubator at 37°C (Kuhl, Flemington, NJ). Eggs were rinsed with 70% alcohol and 3mL albumin was removed. Eggs were windowed and embryos staged according to Hamburger and Hamilton (1951). Embryos at HH st.10 (Hamburger and Hamilton, 1951) were injected with a GFP-encoding vector, pMES, 4.3 ug/mL or pCAX (kind gift of Dr. Cathy Krull, U. Michigan) or pLZRS.gap43.GFP plasmid (kind gift of Dr. Rusty Lansford, Caltech). Fast Green FCF (Sigma, F-7252) at 10mg/mL, was added 1:5 to the injection needle to visualize injection of the construct in ovo. The GFP-plasmid was microinjected into the lumen of the neural tube using a borosilicate glass capillary pulled needle (World precision instruments, MTW100-4) until the region

of the neural tube between the forelimbs and hindlimbs was filled. Constructs were electroporated into pre-migratory NCCs using gold coated Genetrode electrodes (Fisher, BTX512) and an electroporator (Genetronics, San Diego, CA). Eggs were resealed with adhesive tape and incubated at 38°C for 2 days. After this incubation period we evaluated each embryo prior to generating sagittal explants for brightness and uniformity of GFP label using a Zeiss Axiovert microscope with a 10x objective, and selected those embryos that were well labeled and developing normally.

Preparation Of Sagittal Explants

Embryos were removed from the egg using a paper ring (Whatman, #1001185), cleaned in warmed Ringer's solution and the surrounding membranes were carefully removed using forceps. Sagittal explants of embryos ranging from HH st. 17-22 were prepared by using the trunk sagittal explant technique described in Kasemeier et al, (2004) and also briefly described below. A trunk explant is made from the region between the fore and hindlimbs. A cut along the midline of the spinal column is made with a tungsten needle to splay open the spinal cord. A razor blade is then inserted into the incision made with the tungsten needle (A-M Systems, 717000) and sliced through the embryo to produce two sagittal explants that can be cultured medial (cut) or lateral side down.

A Millipore culture plate insert (Millipore, PICMORG50) is prepared by coating with 20 ug/ml of fibronectin (Gibco, 33016-015). To increase image

resolution, we used a modified 6-well dish with a hole in the center of the bottom of the well, and a coverslip sealed with vacuum grease over the hole (Kulesa and Fraser, 1998). The sagittal explant is then transferred onto the Millipore filter with a few drops of Neural basal medium (Gibco, 21103-049) supplemented with B27 (Gibco, 17504-044). The filter with explant is then transferred into the coverslipped well filled with supplemented Neural basal media. Sufficient media is added so that the level of the Millipore filter is reached (for media exchange through the filter to the tissue) but not too much so that the filter floats in the well. The other wells are filled with sterile water and the dish is covered and sealed with parafilm to create a humidified chamber to place on the heated microscope stage.

Time-Lapse Video Microscopy

GFP-labeled explants were visualized using laser scanning confocal microscope (Zeiss LSM). Optical thickness was set between 10 and 20 μm in z-height with a 10x Neofluar (NA = 0.30) lens. This optical thickness was optimal for the observation and tracking of maximal number of cells as they migrated ventrally over longer time periods. The microscope was surrounded with an incubator composed of a snug fitting cardboard box surrounded with thermal insulation (Reflectix, BP24025) and a table top incubator (Lyon Electric, 950-107) fed into one side of the box (Kasemeier et al, 2004). The fluorescent GFP plasmid was excited with the 488nm laser line using the FITC filter. Time-lapse images were recorded every 10 min for an average of between 24 and 36 hours.

Images were digitally collected and analyzed using Zeiss AIM software and ImageJ v1.30 software (developed at NIH and available on the Internet at <http://rsb.info.nih.gov/ij/>). Static image analysis and rendering (depth-coding and embossing) was done using Adobe photoshop 7.0. In total, 12 embryonic explants were imaged for >24 hrs and 5 for between 6-24 hrs. For higher resolution imaging, explants were mounted on a coverslip (as described in Kulesa and Fraser, 2002) and imaged for 4- 6 hrs with a 40x LD-Achroplan, Zeiss objective on a laser scanning confocal microscope (Zeiss LSM Pascal on a Zeiss Axiovert).

Results

To characterize the cellular dynamics during DRG and SG formation, pre-migratory NCCs were fluorescently labeled with a GFP encoding vector and followed using time-lapse confocal microscopy. After allowing sufficient time for GFP expression, transfected NCCs were imaged from a medial (Fig. 3B) or lateral (Fig. 3C) perspective. Imaging from the exposed medial surface in a sagittal explant allows for visualization of NCCs as they migrate ventrolaterally en route to their final destination adjacent to the dorsal aorta (Fig. 3B). Imaging from the lateral perspective offers the ability to follow NCCs from the moment they emerge from the neural tube, form the DRG, and migrate through the rostral half of somites (Fig. 3C). Normal morphology is maintained in the sagittal explant and SG and DRG develop normally through 72 hours in culture. The

uniqueness of the sagittal explant allowed us to follow the complete migratory route of trunk NCCs from the neural tube to their target destinations as they form the DRG and primary chain of SG (Fig. 3A; Movie 1). By analyzing time-lapse confocal imaging sequences, we were able to identify many of the cellular dynamics integral to DRG and SG formation. Here, we describe the spatiotemporal aspects of DRG and SG formation and NCC migratory behaviors not previously discerned from static images.

DRG Formation

The close juxtaposition of the DRG to the neural tube (which are both GFP labeled) can obscure the resolution of the cellular morphological events integral to DRG formation; however, this problem can be largely obviated by imaging the lateral surface of the sagittal explant which provides a relatively clear view of the aggregation of NCCs as they form the DRG anlagen (Fig. 4A). Although the NCCs that give rise to the DRG travel a much shorter distance than those that give rise to the SG, we have identified a few subtle intercellular interactions that mediate DRG formation using time-lapse analysis of sagittal explants. As observed during SG formation (see following results on SG), cross-talk exists between adjacent DRG anlagen as they form, but to a much lesser extent than occurs during SG condensation (Fig. 4B). As cells emigrate from the neural tube, they can extend filopodia across the caudal somite and thereby be in

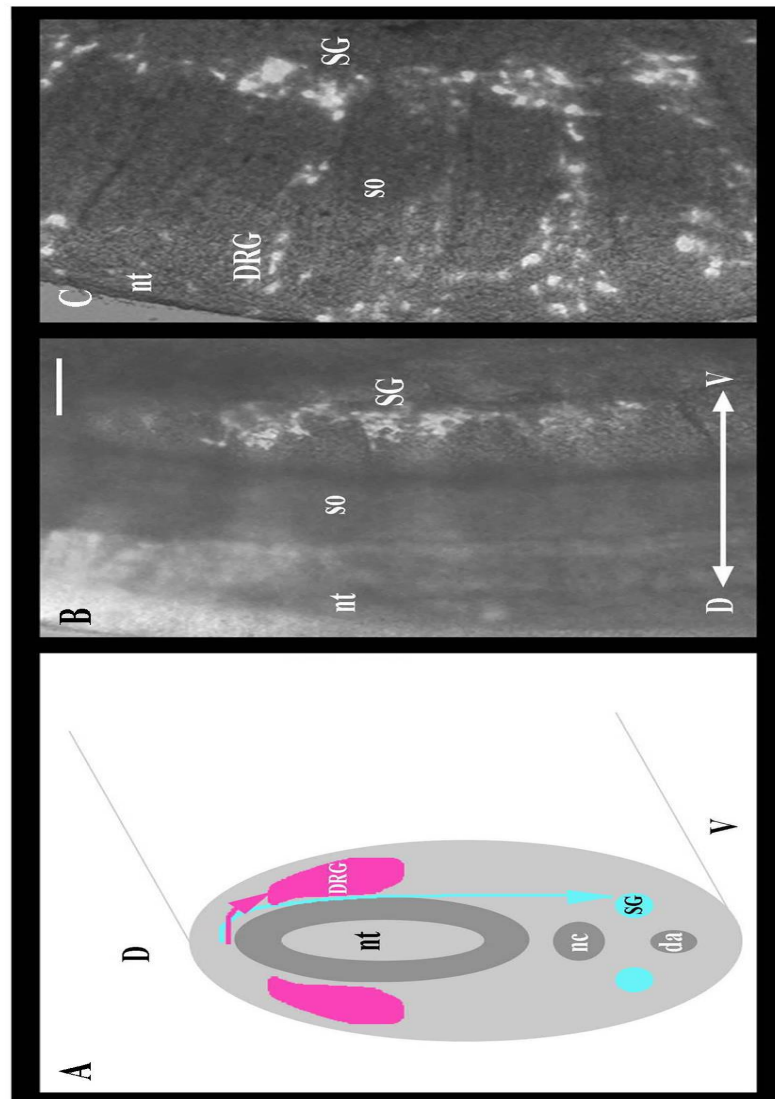


Figure 3. Imaging perspectives in sagittal explants. A. Schematic showing the migratory routes of NCCs from the dorsal neural tube to the DRG (pink) and SG (blue). B and C. Typical views of a sagittal explant containing GFP-labeled NCCs. B. medial view; C. lateral view. nt=neural tube, so=somite, sg=sympathetic ganglia, drg=dorsal root ganglia, nc=notochord, da=dorsal aorta, D=dorsal, V=ventral. Scale bar (B-C)=25um.

contact with cells from two different DRG anlagen. Once the cells commit to one of the condensing DRG, they often continue to reorient and move but to a lesser extent than observed within and between SG anlagen. Cells were observed moving from one DRG anlagen to the neighboring one (Fig. 4C-E) but it was difficult to determine whether cells moved more than one axial level while in the periphery, due to the abundance of cells that were labeled. Cells that switched to the next most caudal or rostral DRG did so while maintaining contact with DRG cells. Cells along the perimeter of the incipient DRG exhibit more movement than cells within the central core. These cells on the perimeter actively extend and retract filopodia into the surrounding environment. Teillet et al (1987), using quail-chick chimeras, also described the repositioning of NCCs along the rostral-caudal axis of the neural tube during DRG formation; we extend those observations by imaging interactions and relocation of cells that occurs once cells have exited the neural tube and are forming the DRG anlagen.

Neural Crest Cells Migrate In Chains

Neural crest cells migrating through the rostral sclerotome form streams of cells interconnected by extensive filopodia and adopt a chain-like formation as they make their way towards the dorsal aorta (Fig. 5). Even though only a subset of NCCs are transfected with the GFP-encoding plasmid, and therefore visible

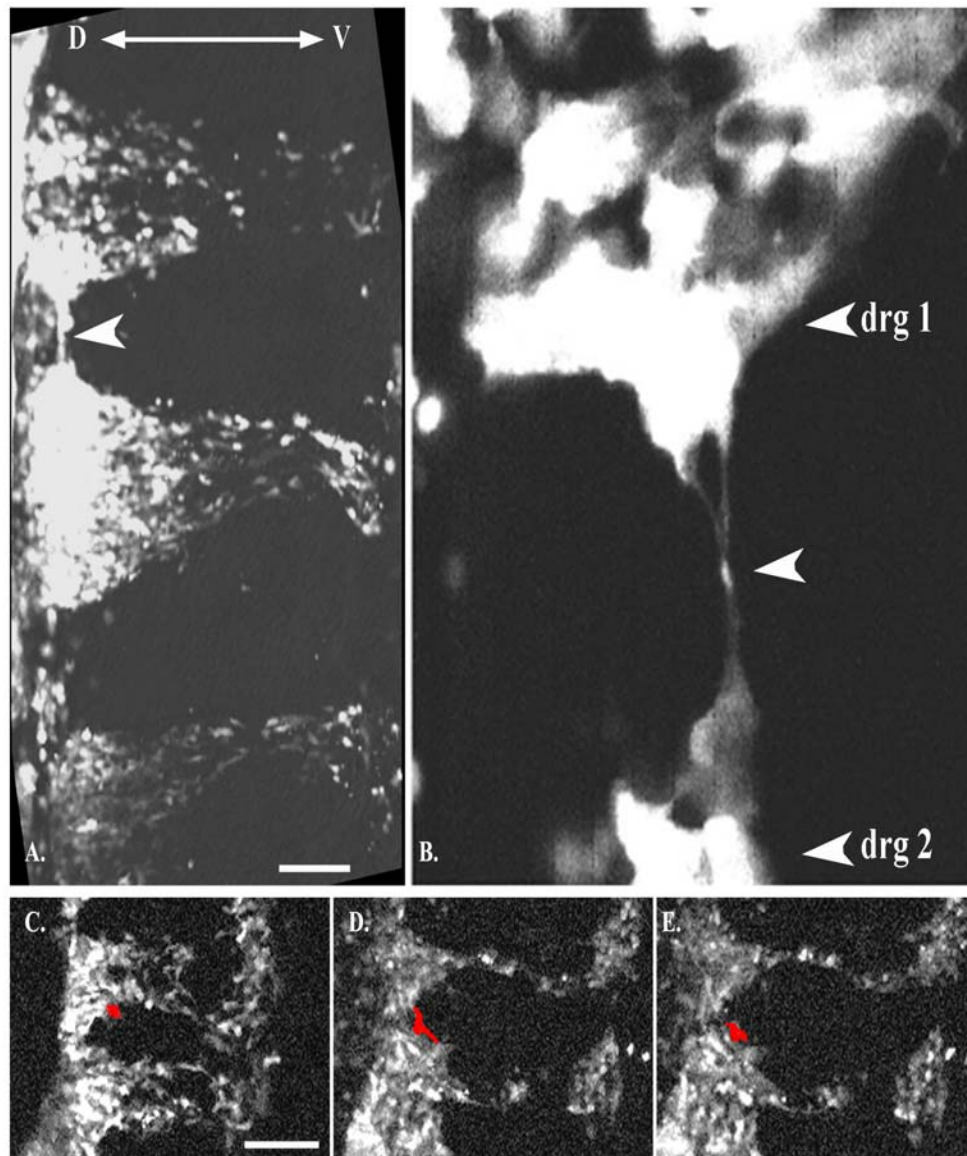


Figure 4. Aggregating neural crest cells as they form the DRG anlagen. Embryos were injected at HH St. 10 with GFP coding vector pMES and sagittal explant mounted at E3.5. **A.** Neural crest cells migrating from 3 axial levels through the rostral somite with arrow indicating cells between two DRG anlagen. Double ended arrow indicates dorsal (D) and ventral (V) directions. **B.** High magnification of area indicated by arrowhead in (A). Cells from DRG1 and DRG2 are in contact with each other. **C-D.** Cell tracking of a single NCC which initially resided in one DRG and then migrated to the DRG immediately posterior. Scale bars, A=20um, C-E=40um.

using fluorescence microscopy, entire chains of labeled NCCs can be resolved that extend from the ventral edge of the DRG, through the sclerotome, to the SG anlagen (Fig. 5B-D). Nearly every fluorescently labeled cell imaged is a member of a chain, indicating that chain behavior is an integral feature of NCC migration through the sclerotome. Cells within a chain extend one filopodium towards a cell in the direction of the SG (ventral) and one filopodium back (dorsally) towards the neural tube (Fig. 5C-D). These filopodia interconnecting a chain of NCCs can change in length as cells migrate, but are always maintained as crest cells migrate towards the dorsal aorta. Virtually all cells observed migrating through the sclerotome maintain filopodial contact with, at minimum, one other cell throughout their migration. Depth coding analysis indicates that the filopodial connections are within a narrow focal plane, ranging from 5-10um with entire chains comprising a focal plane of less than 40um. These chains have been imaged previously at earlier stages in NCC migration (Krull et al., 1995) but not imaged as cells migrate deep ventrally towards the dorsal aorta.

Migration And Reorganization Of Neural Crest Cells As They Form The DRG And SG Anlagen

The retention of filopodial contact between NCCs in a chain appears to be critical for maintaining normal NCC migration. In contrast to what is observed in static images, we find that occasionally NCCs are not completely inhibited from entering and exploring the non-permissive caudal somite. A small percentage of NCCs en route to the dorsal aorta can break from a chain and extend filopodia

into the caudal somite environment (Fig. 6). While exploring the caudal somite, if such cells maintain their original filopodial contacts with their neighbors in the chain, they will continue to migrate in a directed fashion ventrally. These filopodial contacts function to reorient cells that have attempted to break from the chain, apparently by pulling them back into alignment. In rare cases, as shown in the sequence of images in Fig. 6B, a NCC can completely break free from the chain. These cells move extensively in all directions near the chain in an undirected manner and do not re-establish themselves within the chain, nor do they localize to a DRG or SG. Since not all NCCs are GFP+, we can't ascertain whether those cells that break from the visible chain are still in contact with other migrating NCCs. However, we show here that a visible separation from chain neighbors dramatically alters NCC behavior.

At early stages, reorganization of the cellular composition of the DRG and SG anlagen can occur while NCCs are migrating ventrally. A subpopulation of cells migrating in chains towards the dorsal aorta (i.e. site of SG formation) can reverse its ventral trajectory and migrate dorsally (i.e. site of DRG formation; Fig. 7A-D). These cells maintain contacts with their chain neighbors while traveling in have reversed their trajectory can join the DRG anlagen. However, as development ensues, spatial restrictions are imposed on the migratory pattern of trajectory are repelled by an apparent boundary positioned between the DRG and SG anlagen, and are subsequently redirected ventrally towards the SG anlagen (Fig. 7E-G). Simultaneously, some cells within the DRG anlagen that

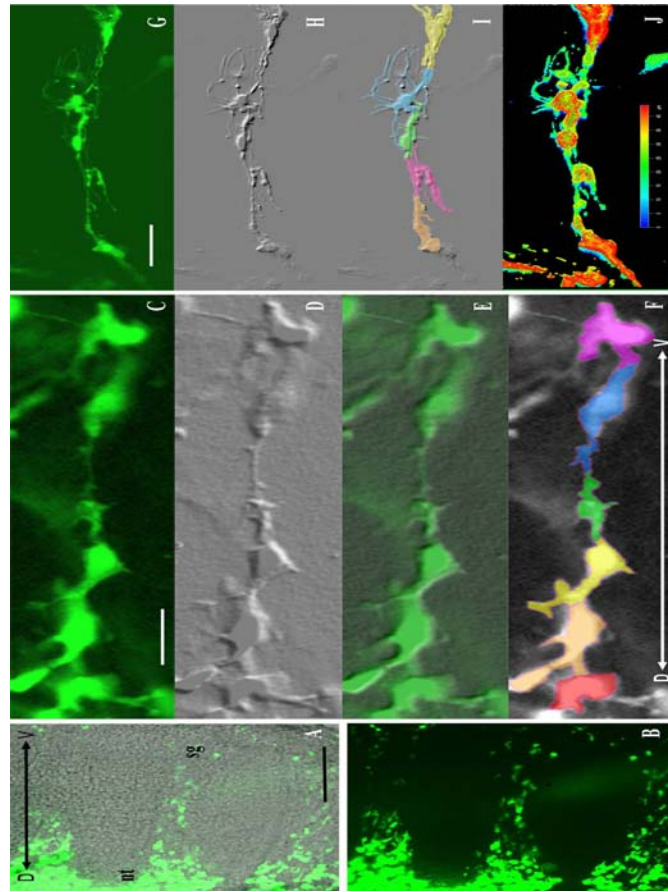


Figure 5. Migrating neural crest cells forming chains through the rostral sclerotome that extend from the DRG to the dorsal aorta. A. Lateral view of sagittal explant of pMES fluorescently labeled NCCs overlaid on brightfield image. B. Fluorescence of labeled NCCs. C-F. Single chain extending from neural tube to dorsal aorta. c. GFP-labeled NCCs. D. embossed image of chain. E. Fluorescence and embossed overlay. F. Individual cells within chain colored separately to define cell boundaries. G-J. Lateral view of sagittal explant of EGFP fluorescently labeled NCCs. G. Fluorescence of single chain. H. Embossed image of chain. I. Individual cells colored separately and overlaid on embossed image. J. Depth coding of entire chain. Arrow indicates dorsal (D) and ventral (V) directions, nt=neural tube, sg=sympathetic ganglia. Scale bars (A-B)=50um, (C-F)=20um, (G-J)=20um.

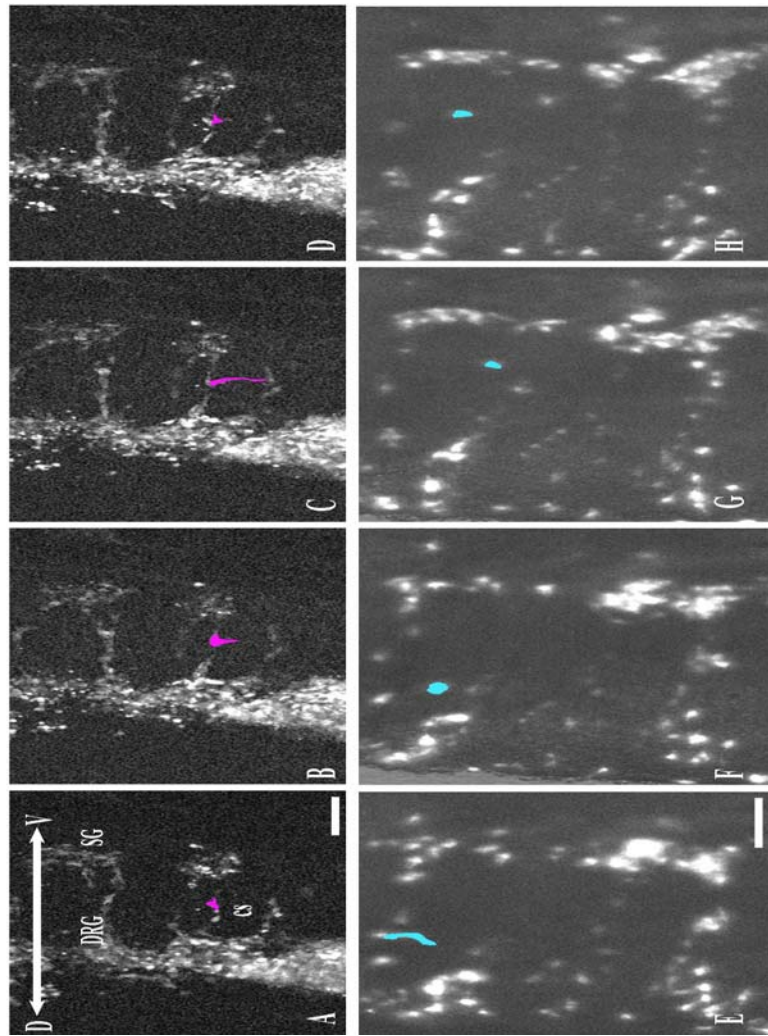


Figure 6. Filopodial contact is important for neural crest migration through the rostral somite. A-D. A cell position midway in a chain extends a filopodia into the caudal somite and touches a cell in a chain in a different axial. The cell maintains filopodial contact with its chain neighbors and returns to it's original chain. E-H. A cell attempts to extend a filopod into the caudal somite but all connections are lost with it's neighbors in the chains. This cell roams through the rostral and caudal somites freely and does not rejoin a stream of NCCs. DRG=dorsal root ganglia, SG=sympathetic ganglia, cs=caudal somite, D=dorsal, V=ventral. Scale bars (A-D)=30um, (E-H)=30um.

the opposite direction. Early on, these previously ventrally-positioned cells that the crest in the ventromedial path. Later migrating cells that reverse their ventral attempt to migrate further ventrally through the somite are also repelled at the same spatially located boundary and restricted from further migration towards the dorsal aorta (Fig. 7H). Thus, spatial constraints within the environment restrict the further exchange of cells between the developing SG and DRG. The developmental stage at which cells can no longer exchange locations dorsally and ventrally corresponds roughly to HH st. 20/21 and was observed in the trunk between the levels of the fore and hind limbs.

Cells that exhibit this reorientation behavior first extend a filopodium in the opposite direction from which they have been migrating. Once that extension is established, the rest of the cell body moves in the same direction as this new extension. We observed cells that reoriented after these extensions were in contact with other GFP+ cells and cases where we couldn't determine whether a cell was contacted or not due to lack of GFP expression in the region contacted by the reorienting cell. The movement is analogous to that of a caterpillar inching along where the head crawls forward and the body then "catches up". At later time points, when cells are incapable of reversing their trajectory, the same cellular behavior is initiated, i.e. extension of a filopodium in the reverse direction, followed by cell movement in that new direction. However, what then ensues is an extension of a new filopodium in the original direction of movement and the subsequent reorientation of the cell back towards its original destination.

Interestingly, the speed of cell movement increases as cells resume their original direction of movement.

Cells Disperse At The Dorsal Aorta

After NCCs successfully traverse the somite, they disperse along a thin corridor rostrally and caudally, bordered on one side by the ventral, outer, sclerotome edge and on the other side by the dorsal aorta (Fig. 8A; Movie 2). The spatial restriction of NCCs to the rostral sclerotome is only maintained until the cells approach the ventral edge of the sclerotome. Once through the sclerotome, NCCs deviate from the metameric pattern that dictated their migration through the somites and instead spread contiguously rostrally and caudally away from their axial level of origin. By tracking individual cells we find that NCCs can move at least 2 segments rostrally or caudally, in agreement with previously published analysis of static images (data not shown; Yip, 1986). However in previous studies, it was not determined how cells ended up at axial segments other than their site of origin: i.e. by moving along the neural tube (site of origin) or the dorsal aorta (target). While our imaging analysis did not focus on NCC behavior while still in the neural tube, our studies clearly indicate extensive migration and reorganization of NCCs once they arrive alongside the dorsal aorta.

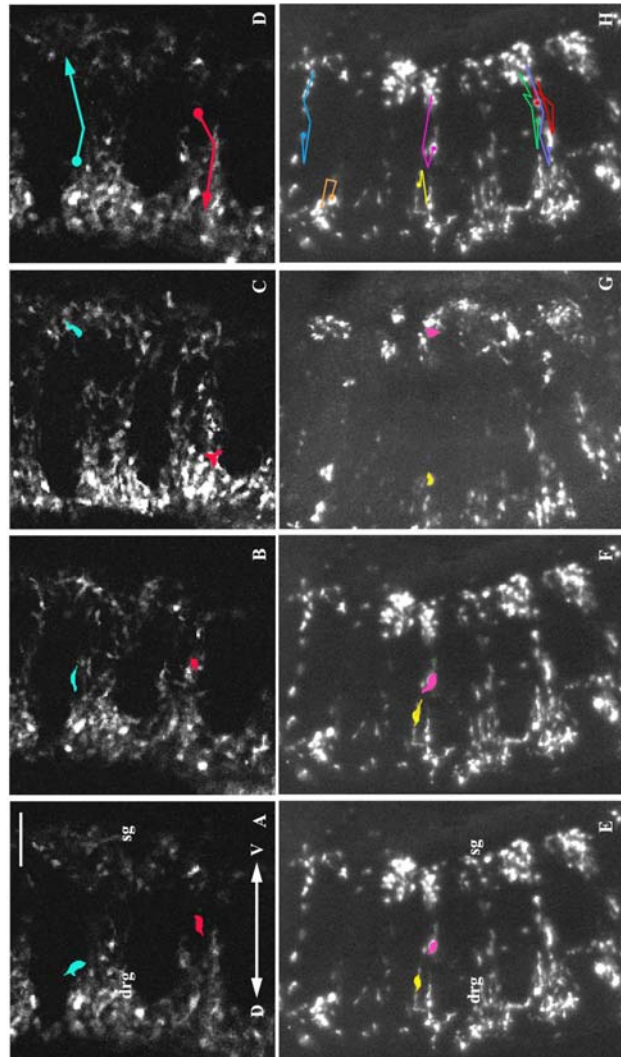


Figure 7. Early migrating neural crest cells, but *not* later migrating cells, can reorient and reverse their initial direction of movement. A-C. Single NCCs tracked in teal and red, were able to reverse their original direction through the rostral sclerotome and populate a different structure than they were originally headed towards. D. Summary of their pathways are shown using vector diagrams. E-H. Cells tracked in yellow and pink try to reverse their direction but are forced back in their original direction. F. Vector diagrams show seven individually tracked cells that were unable to reverse their migration direction. Scale bar=30um, D=dorsal, V=ventral, drg=dorsal root ganglia, sg=sympathetic ganglia.

Discrete SG Arise As A Result Of Active Segregation Of Neural Crest Cells

We have observed two sources of cells that will comprise the SG. One population derives from chains of crest cells that divert from their directed path in the rostral somite, make filopodial contact with cells adjacent to the dorsal aorta, and integrate directly into the inter-ganglionic space between incipient SG anlagen. The second population is derived from chains of crest cells from the neural tube that directly target the SG anlagen, but then move into the surrounding rostral and caudal environment (data not shown). However, in each case, filopodial connections allow cells to enter the inter-ganglionic space that might have been considered inhibitory, based on the known inhibitory properties of the caudal sclerotome (Fig. 8 and 9).

The extensive cellular movement adjacent to the dorsal aorta eventually results in the segregation of NCCs into discrete SG anlagen (Fig. 9; Movies 3 and 4). While resettling, many cells not only alter their direction once, but cells have been tracked that have reoriented themselves and changed their direction of movement numerous times before finally coalescing with their SG anlagen of origin, or a neighboring SG anlagen (Fig. 9 B-G'). We analyzed the location of crest cells during this active period of reorganization and found that roughly 20% of the labeled NCCs were located in between neighboring SG ($21.1\% \pm 3.12$ SD; 289 cells counted) vs. within an incipient SG. While in between SG, cells maintain filopodial contact at all times with other NCCs. By analyzing cell

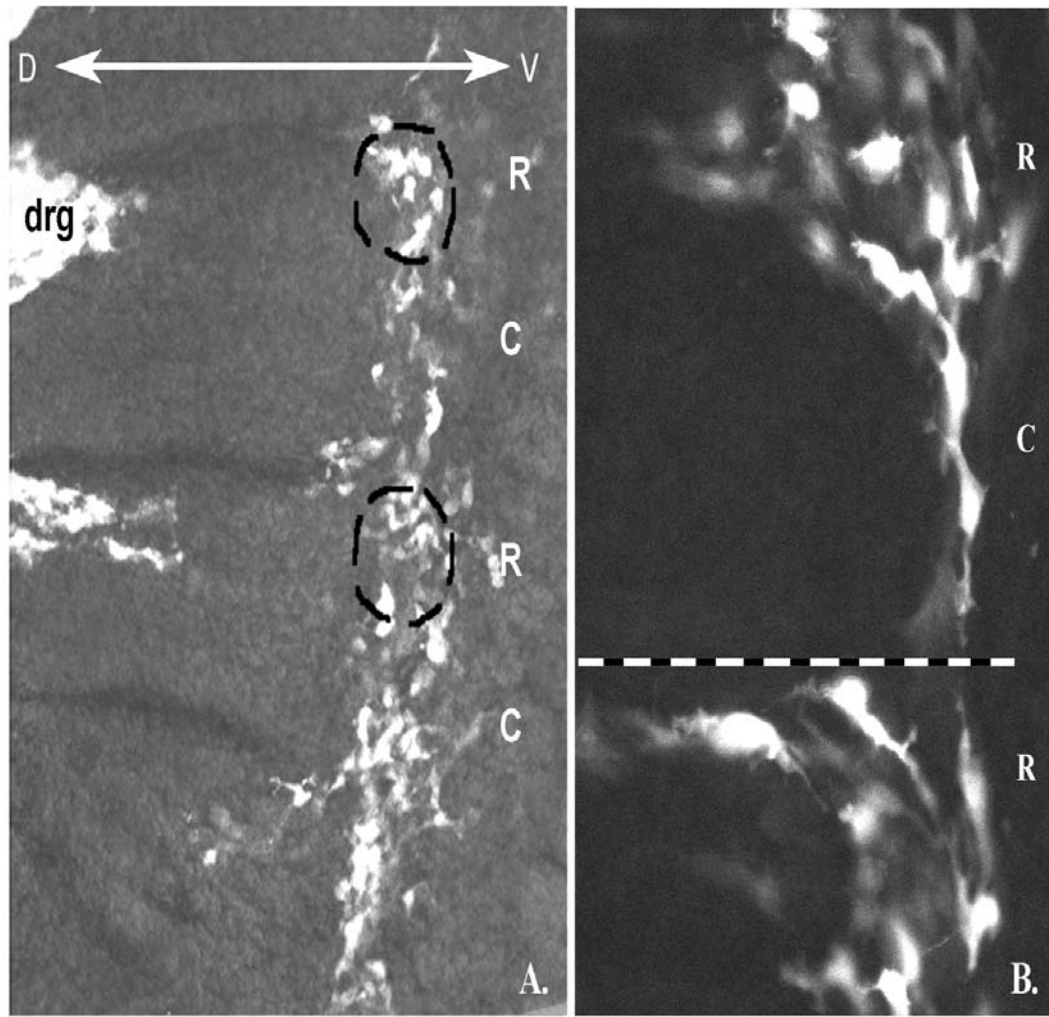


Figure 8. Distribution of neural crest cells as they arrive at the dorsal aorta in a medial explant. Neural crest expressing GFP. A. Medial explant of NCCs initially distributed along the dorsal aorta. Double ended arrow indicates dorsal (D) and ventral (V) directions. B. High magnification view of cells between developing SG at a later time point in this explant. Dotted line indicates somite boundary. R=rostral somite, C=caudal somite, scale bar=25um.

dynamics in Movie 3 & 4, we find that initially NCCs in the inter-ganglionic space extend short filopodia from the entire 360° of cell circumference. However, those filopodia that do not contact a cell in the neighboring SG are much more dynamic and rapidly retracted as compared to filopodia that contact cells in neighboring SG anlagen. Gradually, those filopodia that are not extended towards either of the neighboring SG anlagen become shorter and less frequent than those directed towards neighboring SG. In fact those filopodia that extend in the direction of the SG and contact a cell there tend not to retract and instead thicken and widen as the cell elaborates its contact with the new SG cell. With extensive cell contact to a cell in a neighboring SG, a cell's morphology undergoes rapid phenotypic changes, and in the process alters the extent of its membrane apposition with its neighbors (Figure 10). We have quantified the series of morphological changes undergone by a given cell in the inter-ganglionic space by measuring the extent of its membrane contact with a cell in a neighboring SG, compared to its membrane juxtaposition with another cell in the inter-ganglionic space (Fig. 10B). This analysis reveals that as a cell's membrane association is increased with a cell in the neighboring SG, its membrane apposition with a cell in the inter-ganglionic space is decreased. These morphological changes are not accompanied by "collapse" of filopodia, rather, filopodia are dynamically extended and retracted. Following additional filopodial extensions and contact with cells in the neighboring SG, the cell finally relocates and migrates towards and adheres with the SG while withdrawing its connections with cells in the inter-

ganglionic space. Figure 11 depicts a series of images of a cell migrating from its original axial level, to populate the immediately caudal SG. Sufficient contact seems to be important for cells in this space to be permanently “pulled” into a particular SG. If a cell loses all cell-cell contact with other neighbors in either direction from the cell body, the cell appears to retract all filopodia extending from the cell and migrate with no apparent direction and constantly changing direction and speeds. Therefore, constant intercellular communication mediated by contact, seems to be a key component of the complex sequence of events that lead to the establishment of discrete SG.

Discussion

The goal of this study was to characterize the behavior of trunk NCCs as they migrate and give rise to the DRG and SG anlagen. By labeling premigratory trunk NCCs with a GFP tag, we followed individual cell trajectories from the dorsal neural tube into the ventral periphery in sagittal explants using time-lapse confocal microscopy. With this approach, we have identified several novel and compelling events integral to the formation of DRG and SG that have not been previously described, including the prevalence of chain formations as cells migrate ventrally and their role in maintaining directionality; the fact that NCCs interact extensively and dynamically with neighboring cells as they migrate, and that filopodial contact between neighboring NCCs appears to be critical for maintaining normal migratory routes. Furthermore we show that at early stages,

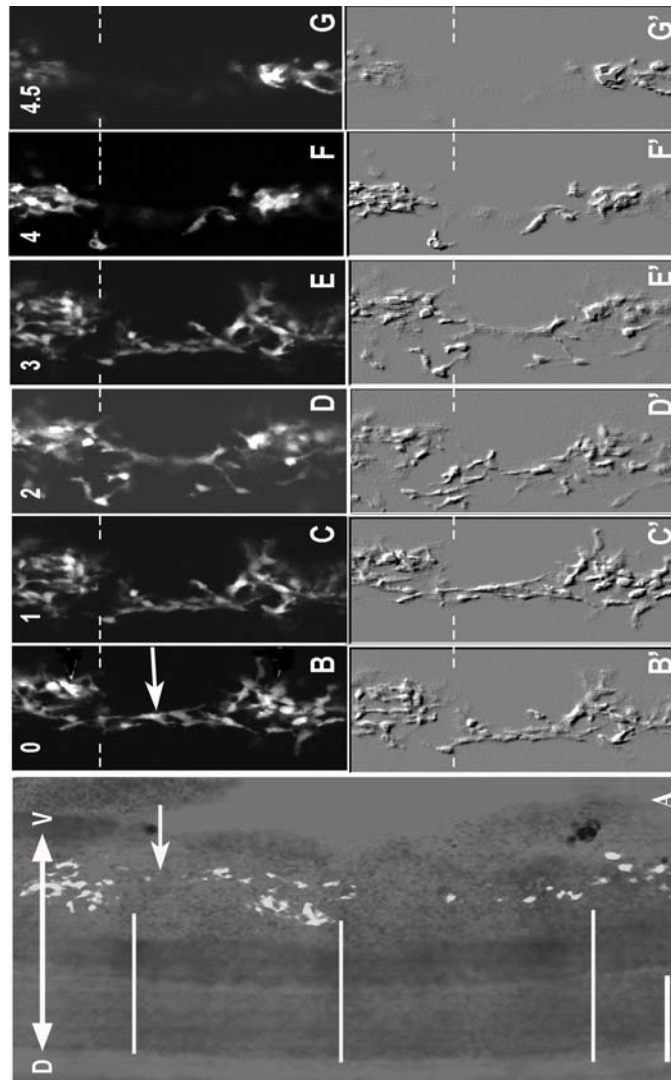


Figure 9. Timelapse analysis of SG formation in chick trunk sagittal explant imaged medially. Fluorescently labeled NCCs in a sagittal explant mounted at E3.5. A. Medial image of NCCs dispersed along dorsal aorta forming the SG anlagen. Solid lines indicate somite borders, double ended arrow indicates dorsal (D) and ventral (V) directions. Inter-ganglionic space and adjacent SG indicated by arrow are shown in B-G and B'-G'. B-G: Segregation of NCCs dispersed along the dorsal aorta into discrete SG anlagen. B'-G' are embossed images of B-G. Dashed lines indicate somite borders. All time is in hours. Scale bar=50um.

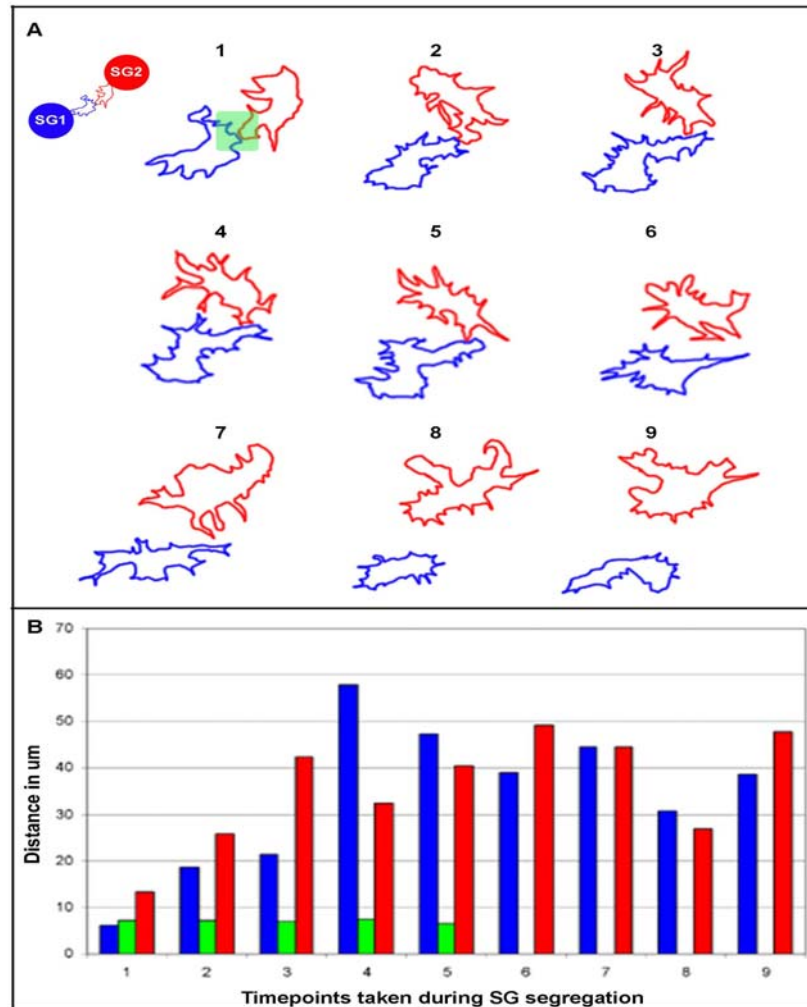


Figure 10. High resolution analysis of single cell dynamics during SG segregation. **A.** Static outline of cells as they segregate into separate SG. Red and blue outlines represent cells imaged at high resolution over time situated in the inter-ganglionic space during SG segregation. The red and blue cells separate and populate two separate SG. Inset shows orientation of cells between SG1 and SG2. Green highlighted region corresponds to the area the green bar measurements were taken in part b. **B.** Amount of cell-cell contact during the segregation process to form discrete ganglia. Blue bars represent the amount of contact (length of cell membrane in um) the blue cell has to SG#1, green bars represent the amount of contact between the red and blue cell in the inter-ganglionic space, red bars represent the amount of contact the red cell has to SG #2.

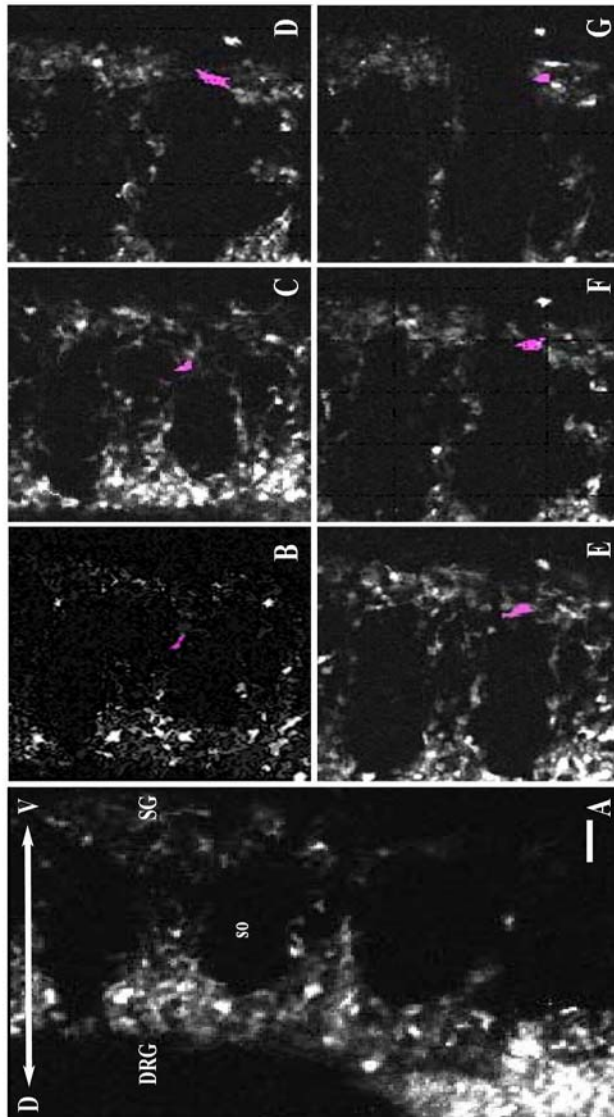


Figure 11. Neural crest cells migrate through the somite and can change axial levels once at the dorsal aorta. B-C. Single cell tracked through time that migrates through the somite at one axial level. D-E. This cell establishes filopodial contact with a cell in the SG anlagen immediately caudal to its axial level. F. Cell moves caudally in the direction of the established contact. G. This cell ends up populating the SG anlagen caudal to its original axial level of migration. D=dorsal, V=ventral, DRG=dorsal root ganglia, SG=sympathetic ganglia, so=somite. Scale bar=20um.

cells located in the DRG and SG anlagen can reverse their migratory direction and switch locations and ganglion type. However, within hours, this plasticity is lost, and cells become incapable of rerouting their trajectory and switching target locations. Most strikingly, we find that the metameric pattern of migration through the rostral somite breaks down once cells reach the ventral outer border of the sclerotome. At this location cells disperse contiguously rostrally and caudally within a narrow corridor between the outer sclerotome border and the dorsal aorta and over the next few hours, through a dynamic process of filopodial extensions/retractions and cell movements, NCCs sort out into discrete sympathetic ganglia.

Chain Formation Is A Common Configuration Adopted By Migrating Cells In The Developing Nervous System

Time-lapse analyses confirmed previously published observations that NCC trajectories maintain a metameric pattern while navigating through the anterior portion of the somites (for review, Krull, 2001; Kuan et al., 2004) and that while migrating, NCCs display collective movements, forming chain-like arrays. Furthermore, the behavior of cells while in the chain, vs. those that apparently break from the chain suggests that cell-cell and cell-environment contacts play a key role in cell guidance to the DRG and SG. NCCs sampled the posterior region of the somite, but maintained stereotypical migratory patterns. Along the migratory route, cells displayed active filopodia in the direction of travel and towards the posterior region of the somite. All cells maintained two long,

prominent filopodial extensions parallel to the direction of travel, connecting them to their chain neighbors ahead and behind. These trunk NCC migratory behaviors of chain migration are reminiscent of those exhibited by migrating cranial and gut NCCs (Kulesa and Fraser, 1998; Young et al., 2004a) and have not been reported in the ventral migration of trunk NCCs as they project towards the dorsal aorta. The chains tended to stretch from the dorsal to ventral edge of the somites and consist on average of approximately five to six cells. Chain migration of neuronal precursors appears to be a common mechanism of cell migration, exhibited prominently by subventricular zone neurons migrating to the adult olfactory bulb in mice (Lois et al, 1996).

Neural Crest Cells Reorganize As They Migrate

Reorganization of cells destined for the DRG and SG not only occurs at the target locations but also while they are en route, as evidenced by our finding of cells being able to reorient ventrally or dorsally from their original location at early time points and contribute to the other ganglion type (i.e. DRG vs. SG; Fig. 5). These data support those of Goldstein and Kalcheim (1991), which indicated the existence of a common pool of NCCs at each axial level that gave rise to both the SG and DRG. Using grafts of exclusively rostral somites, DRG size could be increased which resulted in a corresponding decrease in the SG at the same axial level. Thus, at least a subpopulation of migrating NCCs has the capacity to populate such functionally diverse structures as the DRG and SG, a finding that has also been shown in grafting experiments *in ovo* (Schweitzer et

al., 1983). Reorientation of NCCs was also found in the cranial crest by Kulesa et al. (2000) who showed that hindbrain NCCs can reroute their migratory pathways and compensate for missing NCCs in a neighboring population.

Why do these cells deviate from their neighbors and change their target site? This subpopulation may remain in a more pluripotent, plastic stage longer than its neighbors and hence not be fully committed to one particular ganglion fate. In fact, a temporal pattern is observed in which early on NCCs can change their location from SG to DRG and vice versa, but later are prevented from doing so. As development ensues, the SG and DRG become sufficiently differentiated that a “boundary” is established between the DRG and SG such that NCCs traveling in reverse of their initial migration direction can no longer cross this boundary between these structures and are forced back in their original directions. This may reflect an actual physical boundary, perhaps composed of the known inhibitory proteoglycans that surround the notochord (Tosney and Oakley, 1990; Landolt et al., 1995; Perris and Perissinotto, 2000), and/or be the manifestation of the differentiation of distinct sensory and sympathetic precursors that no longer respond to the local cues in the other ganglion type’s environment and/or become repulsed by cues in the other (now aberrant) environment. Specific members of the basic helix-loop-helix class of transcription factors have been elegantly shown to regulate the differentiation of sensory (*Ngn 1* and *Ngn2*) vs. sympathetic progenitors (*Mash1*) indicating a pre-specification of subsets of migrating NCCs (e.g. Parras et al, 2002; Zirlinger et al., 2002; Luo et al., 2003).

Evidence also indicates a role for Wnt-1 in specifying sensory precursor fate (Lee et al., 2004; Bronner-Fraser, 2004) and for BMPs in inducing the differentiation of sympathetic precursors (Reissmann et al., 1996; Shah et al., 1996; Schneider et al., 1999; McPherson et al., 2000). However, the exact time and place of fate restriction of *migrating* NCCs remains an important question that could be resolved by combining molecular marker methods with time-lapse image analysis.

Formation of Iterated, Discrete Sympathetic Ganglia Is Not The Direct Result Of Patterned Crest Cell Migration Through The Somites

After making the lengthy ventromedial migration and arriving at the site of the incipient SG, NCCs fail to maintain segregated streams, suggesting that the inhibitory factors which restricted the NCCs to the anterior portion of the somite may no longer influence the NCCs once the cells traverse the ventral border of the sclerotome. Instead we show here that individual SG arise as a consequence of extensive NCC reorganization, coalescence and finally condensation into discrete ganglia. Furthermore, this segregation process is mediated by dynamic intercellular contacts and reiterated extension and retraction of multiple filopodia, a behavior that may also be exhibited by neighboring streams of migrating cranial and gut NCC streams (Kulesa and Fraser, 2000; Young et al., 2004a; Teddy and Kulesa, 2004).

Eph/ephrin interactions have been implicated in restricting trunk NCCs to the anterior portions of the somite (Krull et al., 1997; Wang and Anderson, 1997).

Once they have migrated through the ventral border of the sclerotome, the fact that NCCs are then free to spread rostrally and caudally, suggests that these inhibitory molecular mechanisms are no longer operative. Instead, in the absence of inhibitory guidance cues, intercellular interactions among NCCs may dominate. In the cranial region lateral to the neural tube, it has been shown that neighboring NCC streams interact extensively (Kulesa and Fraser, 2000). Thus, if the NCCs relied on intrinsic destination cues from the neural tube, this alteration in behavior from one of strict axial segregation to one of extensive intermixing would not be expected. Instead, our data suggest that the local environment near the site of the incipient SG plays an important role in influencing the behavior of NCCs.

The segregation of NCCs into specific areas along the dorsal aorta to form the SG, suggests a local cell sorting mechanism. NCCs that fill into the areas between the forming SG ultimately coalesce with one of the incipient SG. The mechanism driving the compaction of cells into specific SG may be repulsive and/or attractive. A repulsive molecule(s) may become expressed in the (non-permissive; inter-ganglionic) region between the developing SG that causes the NCCs to move away from the inter-ganglionic space and towards the SG sites. In contrast or in addition, a cell adhesive molecule may be expressed by differentiating sympathetic precursors that induces the neighboring NCCs to adhere and coalesce with the developing SG. Our data clearly indicate that increased intercellular contact with another SG is accompanied by decreased

physical contact with a cell in the inter-ganglionic space. Sorting of NCCs into distinct subpopulations also takes place in the cranial region of the developing vertebrate nervous system. In the hindbrain, Eph/ephrin signals at the rhombomere boundary sites act to sort individual cells into particular rhombomeric segments (Xu et al, 1999). Specifically, some gene expression boundaries appear to correlate with rhombomere boundaries, suggesting a mechanism that fairly precisely marks a border between two cell populations (Xu et al, 1999). The expression of several cell adhesion molecules correlates with the onset of cell coalescence into discrete SG: both N-cadherin and NCAM are expressed in the nascent SG (Duband et al, 1985; Akiyata and Bronner-Fraser, 1992). However, the operative molecular mechanisms mediating the segregation of NCCs into discrete ganglia remain to be elucidated.

Our findings, based on imaging NCCs in their native environment, of the dynamic behavior of NCCs en route to their destination sites adds complexity to the idea that the metameric organization of NCC derived structures depends on the alternation of rostrocaudal properties within the somite. Several classes of guidance molecules have spatiotemporal patterns in the somites consistent with a role in NCCs and/or axon guidance; including semaphorins, neuregulins and BMPs. However, these studies cannot explain our finding of NCC dispersion adjacent to the dorsal aorta followed by crest cell re-segregation into discrete ganglia. What are the molecular mechanisms mediating these distinct behaviors? *In vitro* studies have shown that a class of semaphorins, namely Sema3A,

induces the collapse of sympathetic and DRG growth cones and of migrating NCCs *in vitro* (Adams et al., 1997; Eickholt et al., 1999; Vastrik et al., 1999; Bron et al., 2004). RNA interference of the Sema3A receptor, neuropilin-1, in chick NCCs causes the premature (i.e. dorsal) arrest of NCCs destined to form SG (Bron et al., 2004). The phenotype of mice with targeted deletions of either Sema3A or its receptor neuropilin-1 is complex (Kawasaki et al., 2002). NCCs normally migrate normally through the sclerotome, reach the dorsal aorta and turn on MASH1. However, in mutant mice, they fail to arrest and aggregate at the dorsal aorta and hence to give rise to mature SG. Disruptions in expression of neuregulins and/or their ErbB family of tyrosine kinase receptors, results in severe hypoplasia of the primary sympathetic ganglion chain. Mice with targeted deletions in either the ligand or its receptors, exhibit a lack of neural crest precursor cells in the anlage of the primary sympathetic ganglion chain (Britsch et al., 1998). Although very informative, studies addressing whether these molecules regulate the dispersion and re-segregation of NCCs once they reach the dorsal aorta will be required. The question remains: Why set up a metameric migration pattern when in the end you redistribute and refine at the target location (Young et al., 2004b)?

In summary, timelapse analysis has revealed particularly intriguing, unexpected aspects of DRG and SG formation. The trunk NCC behaviors in chains mimic behaviors reported in cranial and gut NCCs. Surprisingly these tightly segregated streams disperse uniformly once at their target site near the

dorsal aorta, and yet within hours sort out and segregate into discrete SG. What remains is a careful dissection of the molecular mechanisms that mediate the cellular phenomena orchestrating the formation of two of the major neural crest derivatives, the DRG and SG.

CHAPTER 4

INHIBITORY AND ATTRACTIVE MECHANISMS DRIVE
THE EMERGENCE OF SYMPATHETIC GANGLIAIntroduction

Neural crest cells (NCCs) are a migratory population of pluripotent cells that arise at all axial levels of the developing vertebrate embryo at the dorsal midline of the neural tube. NCCs migrate along specific stereotypical pathways through the embryo to generate a wide variety of cell types. Trunk NCCs give rise to melanocytes, glia and neurons of the dorsal root ganglia (DRG) and sympathetic ganglia (SG) and parasympathetic ganglia of the peripheral nervous system (PNS; Weston 1963; LeDouarin 1982; Teillet et al., 1987; Lallier and Bronner-Fraser, 1988). The SG, part of the autonomic nervous system (ANS) of the PNS, which includes the SG, adrenal chromaffin cells, parasympathetic ganglia and the enteric ganglia, provides motor innervation to internal organs, smooth muscle, skin and exocrine glands and is derived completely from NCCs (Weston, 1970; LeDouarin, 1982). The neural crest cells that give rise to the SG migrate ventrally through each somite to reach their target location in the mesenchyme between the dorsal aorta and ventral edge of the somite. At the level of each somite, NCCs migrate through the rostral halves of each somite, forming a repeating pattern of segregated cell migratory streams (Tosney and Oakley, 1990; Krull et al., 1995). Interestingly, after arriving near the SG target

location, the NCCs deviate from the metameric stream pattern and move anteriorly and posteriorly parallel to the dorsal aorta before re-segregating into discrete ganglia (Kasemeier-Kulesa et al., 2005). This second segregation process is intriguing considering that previously it was assumed that the segmented NCC streams through the somites dictated the segmented pattern of the DRG and SG. An important question in biology and also the focus of this paper is to understand how precise spatial patterns of cell types are maintained despite rearrangement of cells both during and after migration to their target location.

Traditional static analyses tracing trunk NCCs has been very informative for characterizing the lineage (Fraser and Bronner-Fraser, 1991; Frank and Sanes, 1991; Zirlinger et al., 2002) and migratory routes of trunk NCCs. After emigrating from the dorsal neural tube, trunk NCCs preferentially migrate through the rostral half of each somite and avoid the caudal half (Keynes and Stern, 1984; Rickman et al., 1985; Bronner-Fraser, 1986). Permissive and inhibitory molecules are responsible for the metameric migration pattern by repelling migrating NCCs away from the caudal somite, and restricting them to the more appealing rostral somite. Several attractive and inhibitory migratory cues are located within the extracellular matrix (ECM). Chondroitin sulfate proteoglycans are specifically expressed in the caudal somite and are repulsive substrates for NCCs (Newgreen et al., 1986; Oakley and Tosney, 1991). ECM molecules such as fibronectin and laminin are permissive substrates for cell migration and are

expressed along the migratory paths of both cranial and trunk NCCs (Thiery et al., 1982; Perris et al., 1993). NCCs express integrin receptors that mediate binding to fibronectin, and in some cases, the cessation of their migration is correlated with the disappearance of fibronectin (Thiery et al., 1982). However, none of the permissive ECM molecules are differentially expressed in the somites, and are unable to influence their metameric pattern.

The Eph/ephrins are a family of developmentally important molecules that mediate inhibitory interactions through complimentary expression patterns of Eph receptors and their ephrin ligands during embryogenesis (Wang and Anderson, 1996; Murai and Pasquale, 2003). EphrinB1 is prominently expressed in the caudal half of chick somites and its receptor EphB2 is expressed on trunk NCCs and inhibits the migration of NCCs through the caudal somite. When either of these molecules is blocked, NCCs are free to migrate through both halves of the somite (Krull et al., 1997). Eph/ephrins are also expressed in complimentary domains in the hindbrain mediating cranial NCC sorting and rhombomere boundary formation (Xu et al., 1999).

In addition to cell-substrate interactions, cell-cell interactions also influence the migratory pattern of NCCs. A well characterized class of adhesion molecules, the cadherins, are transmembrane glycoproteins that mediate calcium dependent homophilic adhesion between cells of same type (Takeichi, 1995). NCCs still associated with the neural tube express N-cadherin, but once they begin to emigrate from the neural tube, they down regulate its expression.

Changes in cadherin expression are associated with changes in cellular morphology and tissue architecture. At the end of their ventral-ward migration, N-cadherin is re-expressed in aggregating cells coincident with the formation of DRG and SG (Bronner-Fraser et al., 1992; Nieto, 2001; Pla et al., 2001). N-cadherin knockout mice die at embryonic day 10 and hence are not amenable for investigation of effects on neural crest migration, formation and segregation of SG but they do display major heart defects and malformations of the neural tube and somites (Radice et al., 1997). N-cadherin is also implicated in several aspects of cardiac development including sorting out of pre-cardial mesoderm and left-right axis specification (Garcio-Castro et al., 2000).

Once at the site of incipient SG, precursors respond to the local secretion of BMP-4 from the dorsal aorta which promotes their differentiation into sympathetic neurons, however, it is not needed for the migration and formation of SG (Reissmann et al., 1996; Shah et al., 1996; Schneider et al., 1999; McPherson et al., 2000). *In vitro* (Erickson and Perris, 1993) and *in vivo* (Bronner-Fraser, 1986) studies have identified a role for neuregulins, semaphorins/collapsins, and the Eph/ephrin family of molecules (Krull, 2001; Graham, 2003) influencing the ventral migration of NCCs. NCCs express the ErbB2 receptor which binds neuregulin-1 and promotes growth and differentiation of neural crest derivatives, including schwann cells and schwann cell precursors (Levi et al., 1995). In ErbB2/ErbB3 and neuregulin knockouts, NCCs do not recognize the migration promoting neuregulin positive areas around the ventral

root and notochord and instead, accumulate ectopically dorsally at the level of the DRG. Hence, due to their distance from the dorsal aorta, and their inability to respond to BMPs secreted from the dorsal aorta they subsequently fail to differentiate into sympathetic neurons (Britsch et al., 1998). Another guidance molecule, Sema3A and its receptor neuropilin-1 which induce among other things growth cone collapse of neurons, are also required for the arrest and aggregation of sympathetic neural precursors at their normal sites adjacent to the dorsal aorta (Kawasaki et al, 2002; Reza et al., 1999). Sema3A is confined to the caudal half of the sclerotome and neuropilin-1 is expressed by trunk NCCs (Eickholt et al., 1999). However, Sema3A or neuropilin-1 knockouts show a normal patterned migration through the rostral somite, suggesting that this signaling mechanism only influences cells once they have completed their migration through the somite.

By tracking live, migrating NCCs as they form SG in the chick embryo, we recently showed that the metameric pattern of the SG is not the direct consequence of segmented migration through the somites (Kasemeier-Kulesa et al., 2005). Our time-lapse analysis demonstrated that although the NCCs migrate through the rostral half of the somite in a segmented fashion, once they reach the ventral, outer surface of the sclerotome and are adjacent to the dorsal aorta, NCCs spread out rostral-caudally and form a continuous stream of cells that runs parallel to the dorsal aorta. With our new understanding of the re-segregation that occurs within the SG at the target site, their overall formation

can be thought of as a two-step process: First the metameric migration pattern through the somites, which gets them to the correct location in the periphery, and secondly, the dispersal and re-segregation at the target site. Here, we are interested in identifying the molecular sorting mechanisms that mediate the segregation of NCCs into discrete ganglia at their target location. To this end, we specifically wanted to analyze NCCs after they have completed their migration through the somite, so as to avoid attributing deficits in segregation to disruptions in migration through the somite. Therefore, knock-out studies involving molecules shown to influence neural crest migration are not useful for discerning the mechanisms that influence the re-segregation of cells along the dorsal aorta corridor. NCCs must make it to their correct location first, before analyzing the secondary re-segregation process. However, little is known whether the identified molecules that influence NCCs through the somites are acting during re-segregation of the SG.

In this study, we investigate the cellular and molecular dynamics that mediate SG formation at the target site. At least two models are possible: segregation may result from an increase in expression of a repulsive molecule in the inter-ganglionic spaces; and/or an increase in expression of an adhesive molecule in NCCs that increases their attractiveness for each other such that they coalesce into discrete ganglia. Here, we provide evidence for both attractive and repulsive mechanisms in the segregation of NCCs into discrete SG. We show that when Eph/ephrin relationships are perturbed, SG do not segregate

properly. In a similar manner, blocking of cell-cell adhesion interactions interferes with the aggregation of discrete SG. Our data suggests that the emergence of the primary chain of SG is dependent on local cell-cell and cell-substratum interactions at their target sites adjacent to the dorsal aorta.

Materials and Methods

Embryos

Fertilized White Leghorn chicken eggs (Ozark Hatchery, Meosho, MO) were placed in a rocking incubator at 37°C (Kuhl, Flemington, NJ). Eggs were rinsed with 70% alcohol and 3mL albumin was removed. Eggs were windowed and embryos staged according to Hamburger and Hamilton (HH; 1951). Embryos at HH st.10 were injected with a GFP-encoding, pMES, 4.3 ug/mL. Fast Green FCF (Sigma, F-7252) at 10ug/mL, was added 1:5 to the injection needle to visualize injection of the construct *in ovo*. The GFP-plasmid was microinjected into the lumen of the neural tube using a borosilicate glass capillary pulled needle (World Precision Instruments, MTW100-4) until the region of the neural tube between the forelimbs and hindlimbs was filled. Constructs were electroporated into pre-migratory neural crest cells using gold coated Genetrode electrodes (Fisher, BTX512) and an electroporator (Genetronics, San Diego, CA). Eggs were resealed with adhesive tape and incubated at 38°C for 2-3 days. After this incubation period we evaluated each embryo prior to manipulation for brightness and uniformity of GFP label using a Zeiss Axiovert microscope with a

10x objective, and selected those embryos that were well labeled and developing normally.

Function Blocking Experiments

Embryos injected with pMES at HH st.10 as previously described were used for these experiments. GFP labeled embryos at E3 were surveyed using a fluorescence dissecting microscope (Carl Zeiss, Stemi SV11). EphB2-Fc (100 ug/mL), ephrinB1-Fc (100 ug/mL), and Fc-control (100 ug/mL) fusion proteins were obtained from R&D Laboratories (467-B2-200, 473-EB-200 and 110-HG respectively). Fast Green FCF (Sigma, F-7252) at 10mg/mL, was added to each fusion protein above at 1:10 to visualize the injection of the fusion protein blocking reagents *in ovo*. EphB2-Fc, ephrinB1-Fc and Fc-control were microinjected immediately lateral to the NCCs that have dispersed in the anterior-posterior axis adjacent to the dorsal aorta. Eggs were re-sealed with adhesive tape and incubated at 38°C for 24-36 hours. Embryos were then removed from the egg (Krull and Kulesa, 1998), mounted on 22x75mm microslides (VWR 48314-024), and imaged using a Zeiss Pascal LSM inverted microscope. NCD2 blocking antibody (300 ug/mL), and Hu rat IgG (control for NCD2; from P. Henion and J. Weston, University of Oregon) were injected and analyzed in the same location and manner as the Eph/ephrin Fc-fusion proteins.

Wild-type chicken N-cadherin (gift of M. Takeichi) and dominant negative N-cadherin, CBR (Riehl et al., 1996), were subcloned into pMES expression vector. Fast Green FCF was added to CBR-pMES (Dominant negative

Cadherin; 4.0 ug/mL) and NCad-pMES (full length N-Cadherin; 3.6 ug/mL) at 1:10. Plasmids were injected in HH st.10 embryos (see above) to label pre-migratory NCCs and embryos were then re-incubated. Embryos were harvested at E3-E4 and SG analyzed.

Static And Time-Lapse Video Microscopy

GFP-labeled explants were visualized using laser scanning confocal microscopy (Zeiss LSM). Optical thickness was set between 10 and 20 um in z-height with a 10x Neofluar (NA=0.30) lens. This optical thickness was optimal for the observation and tracking of maximal number of cells as they migrated over longer time periods. For time-lapse microscopy, the microscope was surrounded with a snug fitting cardboard box and thermal insulation (Reflectix, BP24025) with a table top incubator (Lyon Electric, 950-107) fed into one side of the box (Kasemeier et al, 2004). The fluorescent GFP plasmid was excited with the 488 nm laser line using the FITC filter and all other imaging parameters were as described in Kasemeier-Kulesa et al (2005).

Immunohistochemistry

Embryos were collected in PBS and fixed in 4% PFA for 4 hours. Embryos for cryostat sectioning were rinsed in PBS and run up in a sucrose gradient (5%, 15%, 30% in PBS), embedded in OCT and stored at -80°C. Sagittal cryostat sections were obtained at 10um thickness. Embryos for whole mount staining were fixed as above and washed in PBS, blocked as previously

described (Rifkin et al., 2000) and incubated in primary antibodies for 2-3 days at 4°C. Primary antibodies included: HNK-1 (Developmental studies hybridoma bank, University of Iowa), NCD2 (M. Takeichi, Riken Center, Japan), EphB2 and ephrinB1 (E. Pasquale, Burnham Institute). Embryos were then washed and blocked at room temperature for 1 hour prior to incubation in secondary antibody overnight at 4°C. Embryos were then washed with PBS and stored in 1x PBS at 4°C.

Slides with sectioned embryos for immunohistochemistry were removed from -80°C and rehydrated in TBS for 20 min. Slides were washed with TBS + 0.5% triton x-100 and blocked with TBS + 0.5% triton x-100 + 20% goat serum and 0.01M glucose for 1 hour at room temperature. Primary antibody was applied in blocking solution at 4°C overnight. Slides were then washed and incubated for 1 hour in block at room temperature. Secondary antibody was applied for 1 hour in block at room temperature. Slides were washed and coverslipped using the ProLong Antifade reagent kit (Molecular Probes, P-7481).

Data Analysis

Images were collected, processed and analyzed using Zeiss AIM software. Measurement calculations were performed using AIM software tools. Capturing images using AIM software stores the objective used and calculates in actual micrometers. The program will account and adjust for zoom used in capturing images. Measurements were all standardized using this feature in AIM software.

Results

Dynamic Expression of EphrinB1 Correlates Temporally with NCC Segregation

Our first goal was to identify candidate molecules that could mediate the segregation of NCCs at their target site. Since ephrinB1 and its receptor, EphB2, have been shown to mediate the rostral/caudal polarity of NCC migration through the somite, we tested whether their expression at the dorsal aorta correlated with the changes in NCC behavior at this location. To this end, we obtained antibodies to both ephrinB1 and EphB2 (kind gift of Dr. Elena Pasquale) and examined their expression in the trunk at E2.5-E3.5, the peak period of SG formation in the chick between the forelimbs and hindlimbs. HH st.17 and HH st.20 embryos were collected and prepared for whole mount staining or cryostat sagittal sections. At HH st.17, NCCs are dispersed adjacent to the dorsal aorta in a continuous stream; but by HH st.20, NCCs have re-segregated to form discrete SG (Fig. 12; Kasemeier-Kulesa et al, 2005). Embryos were double labeled with HNK-1 antibody to recognize NCCs and either ephrinB1 or EphB2 antibodies (Fig. 12A-B,D-E). In HH st.17 sagittal sections NCCs are seen dispersed all along the corridor between the lateral edge of the somite and the dorsal aorta (Fig. 12A,B). Expression of ephrinB1 at HH st.17 localizes to the caudal somite as expected, and extends to the lateral edge of the somite but is absent from the corridor in which the NCCs have dispersed (Fig. 12A). EphB2 HH st.17 is expressed by HNK-1 positive NCCs in the rostral somite and on

NCCs that migrated past the somite and have spread along the corridor between the somite and dorsal aorta (Fig. 12B).

By HH st.20, HNK-1 expression indicates that NCCs have segregated and condensed to form discrete ganglia ventral to the rostral half of each somite and are no longer observed opposite the caudal somite (Fig. 12D,E). Interestingly, ephrinB1 expression is still seen in the caudal portion of each somite but now extends past the ventral edge of the somite into the corridor between where the ganglia have formed (inter-ganglionic region; Fig. 12D). EphB2 expression at HH st.20 is still expressed in NCCs in the rostral somite and on the NCCs that have condensed to form the SG (Fig. 12E). Taken together, these results indicate that ephrinB1 expression is dynamic and that initially it is not present in the corridor occupied by NCCs as they disperse rostrally and caudally, however, within the subsequent half-day, its expression extends ventrally and laterally adjacent to the dorsal aorta at the time when SG are segregating and moving out of this area to form discrete ganglia. The receptor for ephrinB1, EphB2, is expressed on the NCCs during this entire period, suggesting that ephrinB1/EphB2 interactions could drive NCCs out of the intervening ganglionic space and ultimately influence the formation of the primary SG chain.

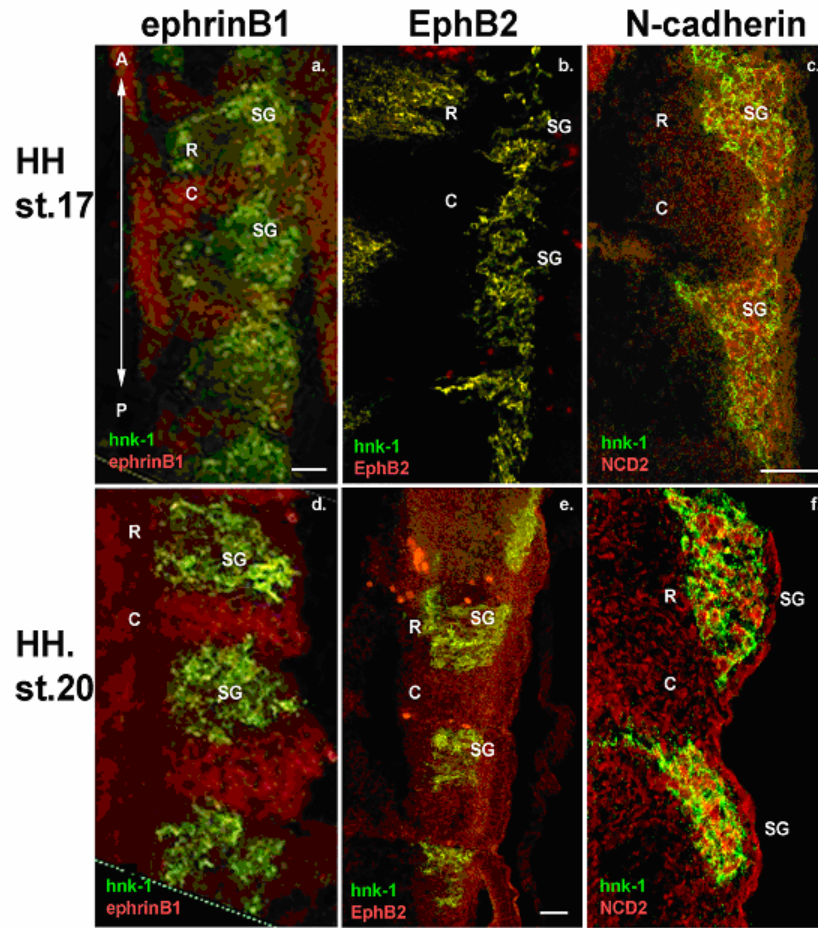


Figure 12. Expression patterns of ephrinB1, EphB2 and N-cadherin during SG formation. Immunohistochemistry performed on HH st.17 and HH st.20 sagittal cryostat sections, 10 μ m. A-C. HH st.17 section. hnk-1 (green) labeled neural crest cells dispersed adjacent to the dorsal aorta. D-F. HH st.20 sections. hnk-1 (green) labeled neural crest cells that have formed sympathetic ganglia adjacent to the rostral somite. A. ephrinB1 (red) expression in caudal somite. Expression ends at rostral edge of caudal somite. D. ephrinB1 (red) expressed in inter-ganglionic region between sympathetic ganglia anlagen. B. EphB2 (red) co-expressed on hnk-1 (green) labeled neural crest cells in rostral somite and dispersed adjacent to the dorsal aorta. E. EphB2 (red) co-expressed on neural crest cells that have formed sympathetic ganglia. C. NCD2 (red) expressed on neural crest cells dispersed adjacent to the dorsal aorta. F. NCD2 (red) expressed in sympathetic ganglia. R=rostral, C=caudal, SG=sympathetic ganglia. Scale bars A-F=20 μ m.

Blocking EphB2 Or ephrinB1 Inhibits Segregation Of SG

Since expression analysis shows ephrinB1 and EphB2 expression at the correct time and location to influence NCCs as they form the primary SG chain, we wanted to test the effects on SG segregation in the presence of blockers of ephrinB1 and EphB2 function. To this end, we obtained ephrinB1 and EphB2 Fc-fusion proteins which compete for binding to their endogenous respective receptor or ligand and hence abrogate signaling that would normally be stimulated by NCCs binding ephrinB1 (Krull et al., 1997). Embryos were injected and electroporated *in ovo* with the GFP-plasmid pMES into the neural tube of HH st.10 embryos to label pre-migratory NCCs. Eggs were sealed and re-incubated for 36 hours. By this stage the NCCs have completed their migration through the somites, reached their future position adjacent to the dorsal aorta and dispersed along the corridor between the somite and dorsal aorta. Using a fluorescent dissecting scope we were able to visualize NCCs using a FITC filter to observe the GFP positive cells. With the egg on the fluorescent dissecting scope we focally injected the Fc-fusion proteins immediately lateral to the cells along the dorsal aorta corridor, injecting a bolus of inhibitor the length of one-half to one somite (Fig. 13a,b). The eggs were then resealed and incubated. After 24 hours, past HH st.20 when the NCCs have formed discrete ganglia, the embryos were removed, mounted on glass slides, imaged on a Zeiss Pascal LSM and the formation of the SG assessed.

In control IgG-Fc injected embryos (n=8), after 24 hours of incubation, discrete SG were seen to form lateral to the rostral half of each somite (Fig.13C). Strikingly, in ephrinB1-Fc injected regions of embryos (n=8), discrete ganglia do not form (Fig. 13D). Instead, GFP positive cells are still seen dispersed along the corridor adjacent to the dorsal aorta and have not condensed into compact ganglia as seen in the control (Fig. 13C). As an internal control, to assess the affects of the inhibitory Fc-Fusion bodies one can analyze the regions of the embryo anterior and posterior to the site of inhibitor injection. Assuming the inhibitor is affecting the NCCs at the axial level at which it was injected, the NCCs anterior and posterior to the level of injection should behave normally. Because the NCCs migrate in a spatio-temporal pattern anterior to posterior within the embryo, the metameric patterning of the SG also form in this order with more anterior SG segregating before those more posterior. By examining SG posterior to the axial level at which ephrinB1-Fc was injected, we found that the SG segregated normally to produce discrete ganglia (Fig. 13D). Taken together, these data show SG segregation is not just slowed down in injected embryos since SG posterior to that level have segregated. Injections of EphB2-Fc (n=8) focally into the region immediately lateral to the NCCs generated very similar results to the ephrinB1-Fc experiments, with a lack of SG segregation (Fig. 13E). In summary, SG do not segregate at the axial levels where either the ephrinB1-Fc or ephB2-Fc inhibitors were injected (Fig. 13).

In addition, we also sought to investigate the behavior of NCCs that were inhibited by the Eph/ephrin blockers in real time. To this end, we injected premigratory NCCs with pMES, and resealed the eggs as before. At HH st.17 we focally injected ephrinB1-Fc lateral to the NCCs when they were near the dorsal aorta. When formation of discrete ganglia should be complete (E3.5), sagittal explants were made and cells along the dorsal aorta were imaged using timelapse confocal microscopy which revealed interesting cellular behavior (Fig. 14; movie 1). Cells remained dispersed continuously adjacent to the dorsal aorta, and were very dynamic with numerous filopodia distributed around the entire cell circumference. Cells positioned in the inter-ganglionic region between SG maintained extensions to cells in the incipient SG anterior and posterior to its position. These cells persisted in this inter-ganglionic region at the time when cells in control embryos have segregated into SG. Cells from the incipient SG region were even observed entering into and residing in the inter-ganglionic region well after normal segmental SG formation should have occurred (Fig.14e-h, asterisk). Cells showed no apparent sign of being inhibited by this territory as they did in control embryos.

N-Cadherin Is Expressed By NCC As They Form SG And Blockade Of Its Function Interferes With SG Coalescence

We also sought to identify which cell adhesion molecules might be expressed by NCCs as they segregate. Based on the extensive evidence for

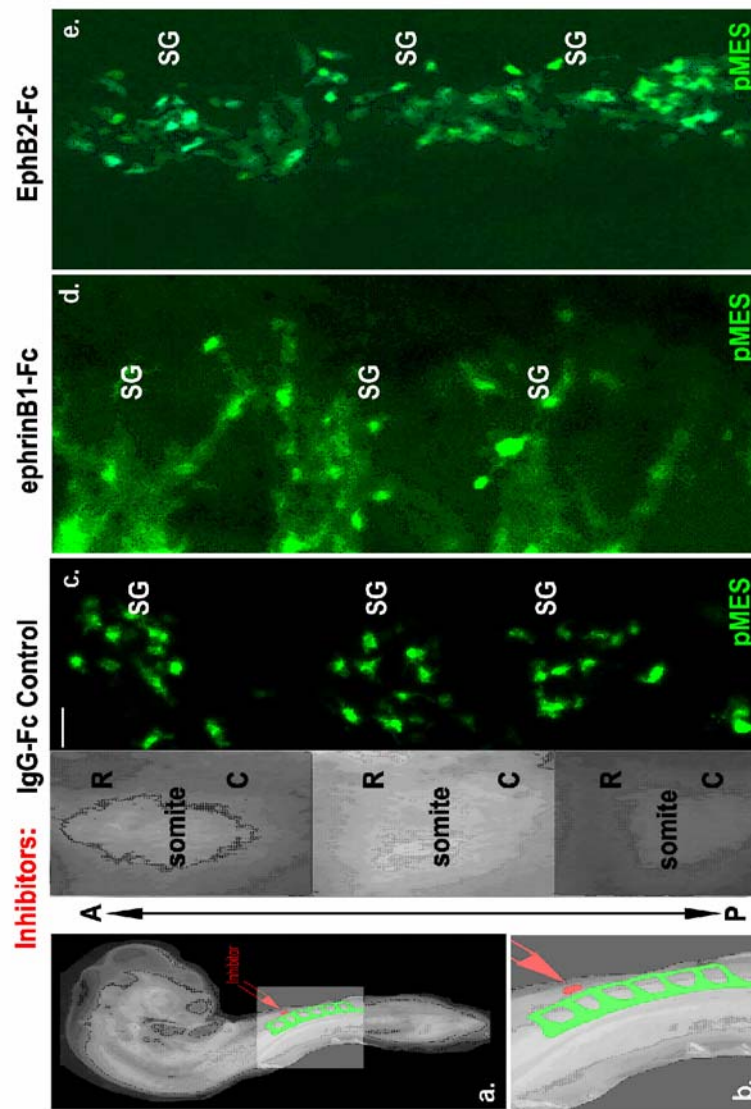


Figure 13. EphB2 and ephrinB1 fusion proteins disrupt sympathetic ganglia formation. A. E3 embryo schematic with GFP labeled trunk neural crest cells. Red arrowhead/needle indicates axial level of fusion protein injection. B. Enlarged region of embryo where inhibitor was injected showing focal delivery of inhibitor the length of 1 somite. C. IgG-Fc control injected embryos with normal sympathetic ganglia forming adjacent to rostral somite. D. ephrinB1-Fc injected embryos. Neural crest cells are found along the anterior-posterior axis of the dorsal aorta in a random orientation. EphB2-Fc injected embryo. Neural crest cells are evenly distributed along anterior-posterior axis of dorsal aorta. SG=sympathetic ganglia, R=rostral, C=caudal. Scale bars=20um.

cadherin regulation of morphogenetic events in embryogenesis, we reexamined the timing of N-cadherin expression in the trunk of E2.5-E3.5 chick embryos during the peak period of SG formation. Our results showed expression of N-cadherin in the nascent SG, in agreement with previous studies of Duband et al. (1985) and Akiyata and Bronner-Fraser (1992) who correlated N-cadherin expression with the onset of cell coalescence into discrete ganglia (Fig. 12C,F).

To determine whether N-cadherin was required during segregation of NCC into SG, we injected a blocking N-cadherin antibody, NCD2, *in ovo* immediately lateral to the NCCs as they were dispersed adjacent to the dorsal aorta, and incubated the embryos for an additional 24 hours (see Eph/ephrin focal injections, Fig. 13A,B). In IgG control injected embryos, normal ganglion formation occurred (Fig. 15A). Analysis of SG formation 24 hours after injection of NCD2 did not immediately indicate any obvious malformation of the primary SG chain. At the axial level of NCD2 injection, SG had segregated and produced discrete ganglia (Fig. 15B). We then measured the length of the SG in the anterior-posterior direction compared to the length of the somite (anterior-posterior) at the same axial level in both control embryos and NCD2 injected embryos. The length (A-P) of the SG was calculated as the percent of somite length it spanned (Fig. 15c). In control, rat IgG injected embryos (Fig. 15a), the length of the SG as compared to the length of the somite was $50.0 \pm 2.5\%$ (n=8;

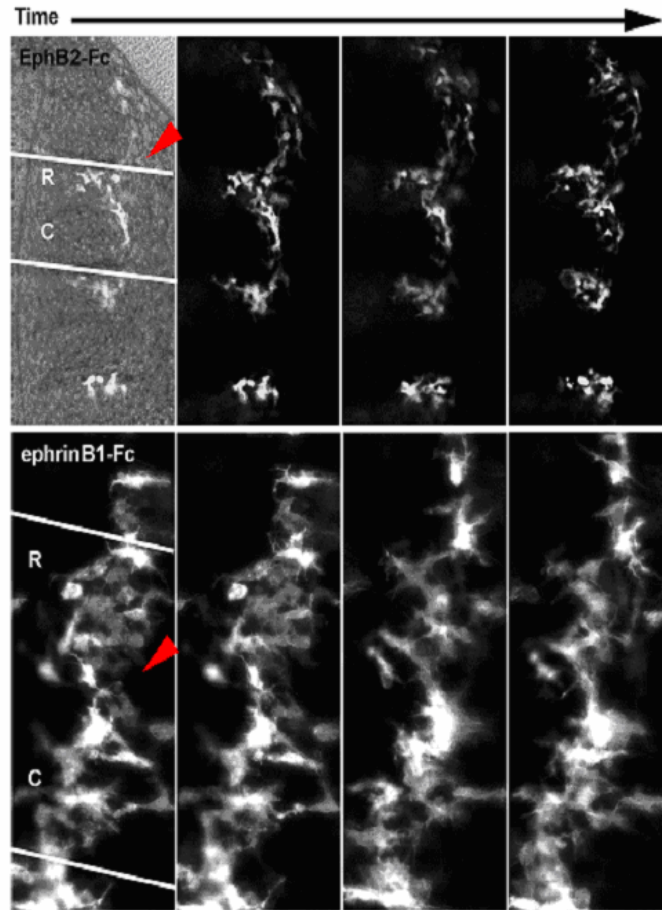


Figure 14. Timelapse analysis of Eph/ephrin Fc-fusion protein injected embryos. Pre-migratory neural crest cells injected with GFP. Fc fusion proteins injected *in ovo* at location of red arrowhead at E3. Sagittal explants made at E3.5 and imaged with timelapse confocal microscopy. Black arrow indicates time increasing from left to right in images. A-D. EphB2-Fc injected embryos. Static images taken from timelapse movie. Sympathetic ganglia at axial level of injection do not segregate but stay continuous along dorsal aorta. Ganglia caudal to axial level of inhibitor injection segregate into ganglia adjacent from rostral half of somite. E-H. ephrinB1-Fc injected embryos. Static images from timelapse reveal neural crest cells remain continuous adjacent to dorsal aorta and do not form discrete ganglia at axial level of inhibitor injection. R=rostral, C=caudal. Scale bars=20 μ m.

Fig. 15E). In NCD2 blocker injected embryos, the length of the SG increased to $70.5 \pm 4.9\%$ (n=18) of the length of the somite (Fig. 15F). Thus, the ganglia were less tightly aggregated in the presence of NCD2 than in control embryos.

As another approach to interfere with N-cadherin function, we injected a dominant-negative cadherin (DN-cadherin) and full length N-cadherin (FL-cadherin) constructs in the pMES vector containing a GFP reporter into HH st.10 embryos, and incubated for 48 hours. The dominant negative cadherin construct contains the catenin-binding regions of N-cadherin, which has been previously shown to function as a dominant-negative construct, inhibiting N-cadherin function *in vivo* (Riehl et al., 1996; R. Bradley, data not shown). Embryos were harvested, mounted on glass slides and imaged using a Zeiss Pascal LSM microscope. To analyze the SG, we measured the length of the SG in comparison to the length of the somite at the same axial level (Fig. 15E) in pMES, DN-cadherin and FL-cadherin embryos. In DN-cadherin injected embryos, NCCs were able to segregate into discrete ganglia but SG size increased in length as in the NCD2 blocker embryos (Fig. 15E). In control pMES injected embryos, the length of the SG was $46.6 \pm 4.1\%$ (n=10) of the length of the somite. In DN-cadherin embryos the length of the SG increased to $75.8 \pm 4.6\%$ (n=11) of the length of the somite. This increase in somite span is comparable to the increase that resulted from the blocking NCD2 antibody injections. Interestingly, FL-cadherin injected embryos had a subtly different

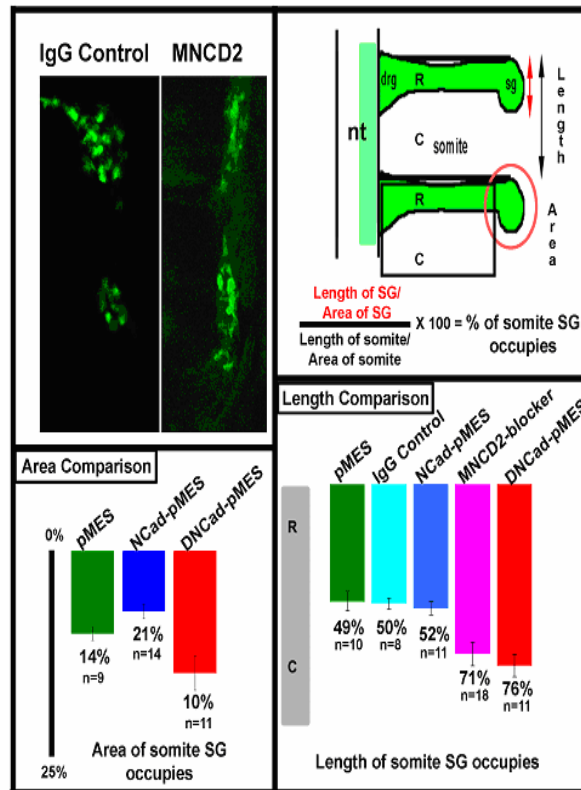


Figure 15. Disrupting N-cadherin adhesion in neural crest cells alters sympathetic ganglia formation. A-B. GFP labeled trunk neural crest cells in anlagen of sympathetic ganglia in E3.5 embryos. A. IgG control injected embryo with discrete sympathetic ganglia formation. B. NCD2 *in ovo* injected embryo lateral to neural crest cells adjacent to dorsal aorta at E3. Embryo imaged at E3.5. Discrete ganglia formation observed but with increase in length of ganglia as compared to control injected embryo. C. Schematic showing length and area comparisons of size of SG compared to size of somite at the same axial level in injected embryos. D. Area comparison of pMES, FL-cadherin and DN-cadherin injected embryos imaged at E3.5 after sympathetic ganglia formation is complete. E. Length comparison of sympathetic ganglia in pMES, IgG control inhibitor, FL-cadherin-pMES, NCD2 inhibitor and DN-cadherin-pMES injected embryos at E3 and imaged at E3.5. nt=neural tube, R=rostral, C=caudal, sg=sympathetic ganglia, drg=dorsal root ganglia, green bar=pMES injected neural crest cells, light blue=IgG control injected blocking antibody into pMES labeled embryo, dark blue bar= FL-cadherin=pMES injected neural crest cells, NCD2 blocking antibody injected into pMES labeled embryos, red bar=DN-cadherin-pMES injected neural crest cells. Scale bars=20um.

phenotype than control embryos. The majority of FL-cadherin positive cells segregated into ganglia adjacent to the rostral half of each somite, spanning $52 \pm 2.9\%$ ($n=14$). However, there was a subpopulation of cells that maintained their connection across the inter-ganglionic regions between SG, with filopodia contacting the ganglia rostral and caudal to its position. This subpopulation of cells did not form a dense stream between ganglia but rather filopodial connections between adjacent ganglia one cell in length.

We next compared the area occupied by the SG in relation to that of the somite, in 2D (Fig. 14D). Due to inherent size differences of somites and ganglia at different axial levels, the SG area was calculated as a percent of the total area of the somite at the same axial level. The SG in pMES injected embryos occupied $14.2 \pm 1.17\%$ ($n=9$) of the area of the somite (Fig. 15A). The ratio of SG area in DN-cadherin embryos was 21.0 ± 2.93 ($n=14$, $p=0.004$), a 48% increase in SG size as compared to pMES embryos (Fig. 15b). FL-cadherin embryo SG size ratio was $10.4 \pm 1.26\%$ ($n=11$, $p=0.001$), a 27% decrease in SG size compared to pMES embryos (Fig. 15C). Thus, interfering with N-cadherin function in NCCs alters their cell adhesion capacity and their ability to segregate into discrete ganglia (Fig. 15D,E); an increase in N-cadherin function decreases ganglion size while a decrease in N-cadherin function causes an increase in ganglion size.

In addition to static analysis of SG sizes, timelapse confocal imaging was used to assess cell dynamics in the presence of DN-cadherin and FL-cadherin as

compared to pMES. Cells migrating along the dorsal aorta in pMES injected embryos migrated at an average speed of 62.1 ± 15.3 $\mu\text{m/hr}$ ($n=10$). However, cells in FL-cadherin injected embryos moved 74% slower at an average speed of 16.2 ± 2.4 $\mu\text{m/hr}$ ($n=12$; $p<0.001$). Thus, cells in FL-cadherin injected embryos moved 53% slower than pMES cells. However, DN-cadherin cells moved at an intermediate speed of 29.2 ± 3.5 $\mu\text{m/hr}$ ($n=14$; $p<0.000$). Examining the morphology of individual cells of pMES, FL-cadherin or DN-cadherin also revealed differences in number of filopodia (Fig. 16; counting only those filopodia over 1 μm in thickness). FL-cadherin cells possessed numerous filopodia, 6.1 ± 0.91 , extending and contacting cells in all directions (Fig. 16e,f), whereas pMES cells tended to have fewer filopodia ($3.6 \pm .79$) but were longer and thicker in nature than FL-Cadherin cells (Fig. 16A,B). Strikingly, DN-cadherin cells had many fewer filopodia (1.9 ± 0.6) than pMES cells and mostly extended in a bipolar direction from the cell (Fig. 16C,D).

Discussion

The patterned migration of NCC through the sclerotome has long been thought to be the basis for the metameric pattern of the DRG, SG and spinal motor axons (Bronner-Fraser, 1986; Lallier and Bronner-Fraser, 1988; Oakley and Tosney, 1993). Recently, we have shown using timelapse analysis that upon arriving adjacent to the dorsal aorta, NCCs spread out rostrally and caudally, thereby intermixing their patterned migration through the somite. Then

they undergo a second, re-segregation process to form the chain of discrete primary SG (Kasemeier-Kulesa et al., 2005). Thus, the SG do not emerge as a direct consequence of metameric migration through the somite, but rather due to local cues as the target. Our goal here was to identify the mechanisms that mediate this sorting process at the target site. The results in this study present two mechanisms that pattern the formation of the primary SG (Fig. 17). The first mechanism involves the inhibition of cell migration in the inter-ganglionic space via activation of Eph/ephrins. EphB2 receptors on NCCs and the emergence of ephrinB1 ligand expression in the domains adjacent to the ventral edge of the caudal somite inhibits the NCCs from remaining in the region ventral to the caudal somite, forcing the NCCs to accumulate in the regions immediately adjacent to the rostral somite that is devoid of ephrinB1. Secondly, cells are influenced within a regional domain via homophilic adhesion molecules. As the NCCs accumulate in closer proximity to one another (migrating away from the ephrinB1 positive regions), N-cadherin expressed on these cells signals them to adhere to one another. These two distinct behaviors, inhibitory and adhesive mechanisms, ultimately work together to pattern the primary SG chain.

Eph receptors and their ephrin ligands are key players in controlling cell movements in many tissues and at multiple stages of patterning through their ability to form tissue boundaries and segregate cells. In addition to restricting trunk NCC migration to the rostral half of each somite (Krull et al., 1997; Wang and Anderson, 1997), Eph/ephrins also play key roles in repulsive guidance of

axonal growth cones (Pollakov et al., 2004). Xu et al. (1999) showed that in addition to cell adhesion molecules, activation of Eph receptors or ephrins can also drive cell sorting in the rhombomeres of the zebrafish. Our results identify the need for both adhesive and repulsive mechanisms in ganglion formation agreeing with Xu's findings that adhesive systems could be coupled with Eph/ephrins to lead to cellular organization.

How important are EphB2/ephrinB1 interactions during the formation of the primary SG chain? In our experiments we blocked EphB2 and ephrinB1 while NCCs were continuous in the corridor adjacent to the dorsal aorta. Our results from these experiments demonstrate that SG formation is severely affected. In the absence of EphB2 and ephrinB1 signaling, discrete SG do not form. Cells remain continuously distributed along the dorsal aorta with no apparent formation of ganglia. Dense streams of EphB2 expressing NCCs migrate through the entire rostral, ephrinB1 devoid sclerotome, with cells migrating immediately adjacent to the caudal somite boundaries. Once they migrate past the ventral edge of the sclerotome they encounter an ephrinB1 devoid region and disperse in the anterior and posterior direction. Blocking the inhibitory interactions of the Eph/ephrins that normally drives NCCs away from the inter-ganglionic space, keeps the NCCs dispersed along the dorsal aorta not

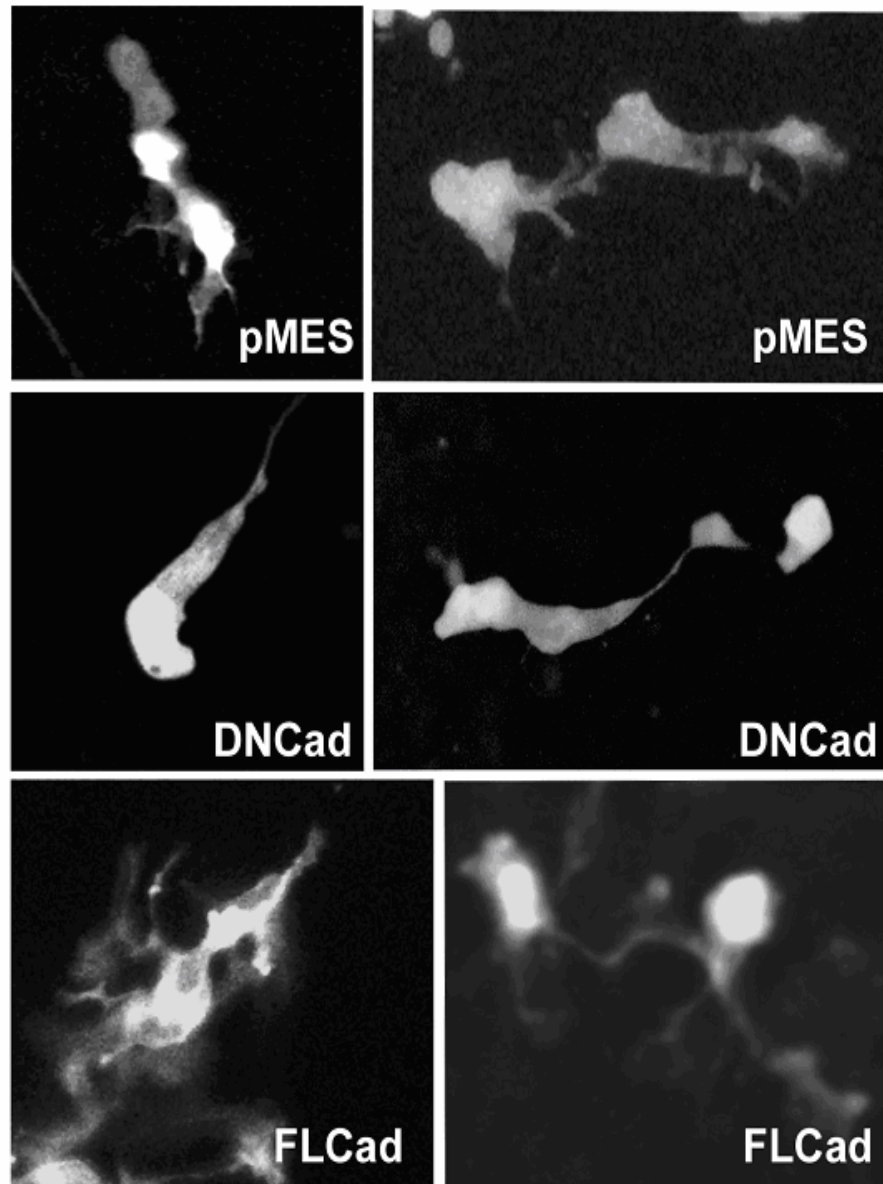


Figure 16. Cell shape comparison in pMES control, DN-cadherin and FL-cadherin expressing neural crest cells. Pre-migratory neural crest cells labeled in HH st.10 embryos. Cells imaged at E3-E3.5 during sympathetic ganglia formation adjacent to the dorsal aorta. A-B. pMES control expressing neural crest cells. C-D. DN-cadherin expressing neural crest cells. E. FL-cadherin injected embryos at E3.5 after sympathetic ganglia formation with connections maintained between ganglia. F-G. FL-cadherin expressing neural crest cells. Scale bars=20um.

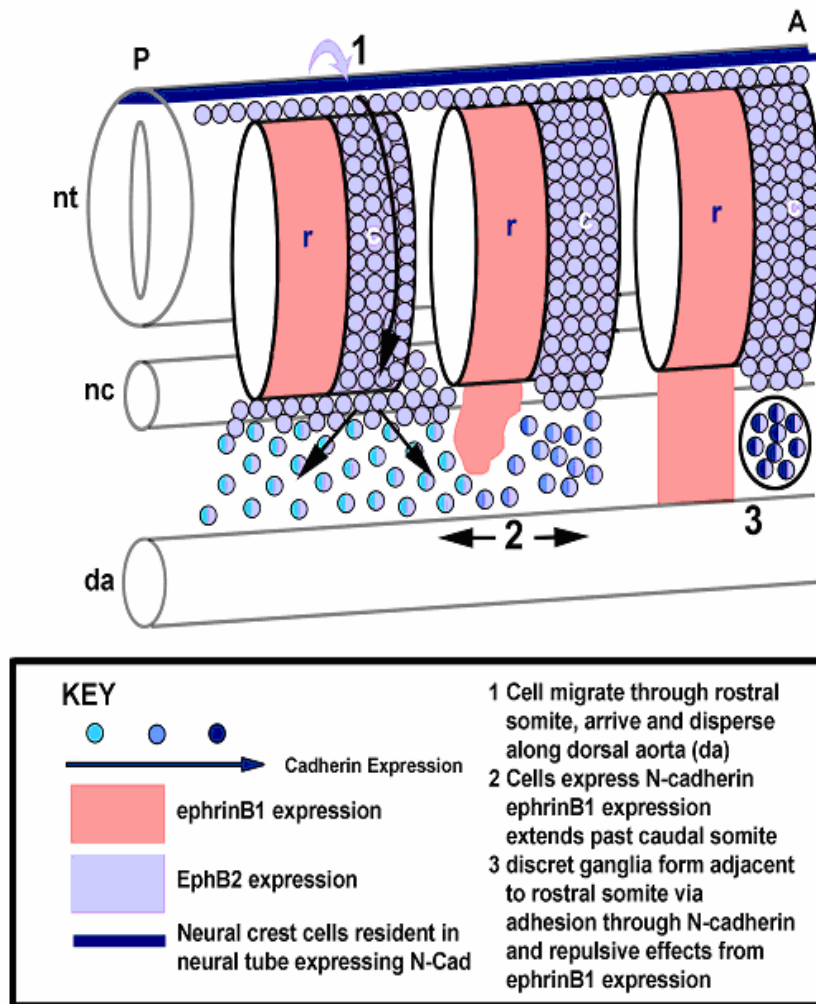


Figure 17. Model of events for proper sympathetic ganglia formation. Schematic model showing important events in a spatio-temporal pattern for discrete sympathetic ganglia formation adjacent to ventral edge of rostral somite. 1. Neural crest cells migrate from neural tube through rostral somite. 2. Neural crest cells disperse adjacent to the dorsal aorta. ephrinB1 expression established in inter-ganglionic regions between developing ganglia. 3. N-cadherin adhesion and inhibitory ephrinB1 expression induce formation of discrete ganglia. Purple=EphB2 expression, blue=N-cadherin expressing neural crest cells, pink=ephrinB1 expression. A=anterior, P=posterior, r=rostral, c=caudal, nt=neural tube, nc=notochord, da=dorsal aorta.

in close enough proximity to each other for N-cadherin to drive ganglion formation.

On the other hand, how important are adhesion molecules during SG formation? By blocking N-cadherin, as cells are dispersed adjacent to the dorsal aorta normal compact SG formation fails to occur. The NCCs migrate out of the inter-ganglionic region where the inhibitory interaction between Eph/ephrins occur. Most migrate within the area lateral to the rostral somite but some stay extended lateral from the caudal somite and form larger ganglia, potentially due to a decrease in cell adhesion from mis-expression of N-Cadherin function.

Timelapse analysis of FL-Cadherin and DN-Cadherin embryos reveal striking differences in cell velocities. FL-Cadherin cells presumably possess more N-Cadherin on their surface and thus increasing the potential to form homophilic adhesions with other cells expressing N-cadherin. Thus, the more cell-to-cell contacts, the more slowly they move. However, one might predict that with fewer functional cadherin molecules expressed on a cells surface, these cells would move faster than control cells, but in fact they don't. Pla et al (2001) showed that dissociated, isolated NCCs in culture did not migrate, suggesting that cells may need a "baseline" of contacts with one another to migrate and below this threshold they do not move, due to lack of communication with their neighbors. We can conclude from these experiments that the cell adhesion exerted through N-cadherin is not sufficient by itself to elicit proper primary SG chain formation.

N-cadherin has also been shown to influence cellular condensation in cartilage formation (Tuan, 2003). Since N-cadherin is expressed in multiple tissues at several time points in the developing embryo, it is not surprising that it can act during two stages of NCC development: First, in neural tube formation and later to promote the aggregation of SG. Studies have also shown, for mixed cell populations expressing different cell adhesion molecules, that differential expression of cell adhesion molecules can lead to population sorting (Friedlander et al., 1989; Nose et al., 1988). In our system, we are presumably dealing with one cell type during the sorting of NCCs into discrete ganglia and our data do not preclude a role for other adhesion molecules in the aggregation of NCCs as they form SG. There is currently no evidence to support a single cell adhesion molecule capable of forming a patterned structure such as the metameric pattern of the primary SG chain. Taken together our data indicate that cell adhesion and inhibitory mechanisms work together to sort SG into discrete ganglia.

In our previous study we showed that the segregation of cells into discrete SG is a very dynamic process involving the reiterative extension and retraction of multiple filopodia and intercellular connections (Kasemeier-Kulesa et al., 2005). As cells separate to form discrete ganglia, they continuously change their shape such that we could measure a pronounced corresponding increase in their membrane area juxtaposition with cells in the adjacent SG. Thus membrane-membrane interactions are obviously playing a major role in causing cells to aggregate and leave the inter-ganglionic space. Our data here demonstrate that

this membrane adhesion is necessary for coalescence of ganglia and that N-cadherin mediates the tight juxtaposition and aggregation of sympathetic precursors.

One important question raised by our findings is why do the NCCs fated to form SG need to re-mingle with themselves once they arrive at their target location and undergo a second round of segregation after their metameric migration through the somite? This inter-mingling of segregating NCC streams has also been observed in cranial NCCs. Brachial arch streams are set up by populations that remain segmented on their way to the brachial arches. Kulesa and Fraser (2000) showed that the trajectories of individual NCCs are much less stereotyped than may have been assumed and demonstrated that distinct streams of cells moving towards the brachial arches do not form strictly from segregated migration behavior. They show that NCC streams from the second and third brachial arches exhibit significant interactions after they migrate lateral to the otic vessel (Kulesa and Fraser, 2000). In the hindbrain, cranial NCCs form streams exiting adjacent to rhombomeres (Lumsden and Keynes, 1989) to give rise to a characteristic pattern of densely populated areas separated by NCC-free zones. Similar to trunk NCCs, in the cranial region, NCC streams have been shown to interact laterally from the neural tube (Kulesa and Fraser, 2000).

One hypothesis could be that the metameric pattern through the somites functions as a crude mechanism to get the cells to the correct location in the embryo. Cells may first need to reach the dorsal aorta to respond to local cues in

the environment to obtain instructions for their differentiation. They then re-segregate to ensure that each SG has the correct populations and numbers of cells. In support of this, by the time sorting occurs at the dorsal aorta, there tends to be an asymmetry in the distribution of NCCs, with the majority gathered adjacent to the rostral somite, as a direct result of patterned migration through the somite. Thus they perhaps serve as a “critical mass” that can then attract the cells in the inter-ganglionic space as they tightly coalesce into discrete ganglia. Thus the emergence of discrete SG is a two step process, with NCCs that reside in the inter-ganglionic space driven by two distinct molecular interactions: one that tends to drive them away from the inter-ganglionic space via inhibitory ephrin/Eph receptor interactions, while simultaneously cells that contact cells in the adjacent SG increase their adhesion with them and ultimately fuse with the incipient SG.

Our data indicates the need for both adhesive and repulsive mechanisms in the emergence of discrete SG (Fig. 16). If cells were strictly following the ephrinB1 boundary instructions, there would be no cue driving NCCs to form compact ganglia. If the cells were solely responding to the N-cadherin mediated adhesion there would be no instructions for them to segregate into a metameric pattern throughout the axis of the embryos. Thus, as in many other developing tissues, cells integrate both attractive and repulsive cues as they sort to form embryonic structures.

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