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Fate map and morphogenesis of presumptive neural crest and dorsal neural tube

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ABSTRACT

In contrast to the classical assumption that neural crest cells are induced in chick as the neural folds elevate, recent data suggest that they are already specified during gastrulation. This prompted us to map the origin of the neural crest and dorsal neural tube in the early avian embryo. Using a combination of focal dye injections and time-lapse imaging, we find that neural crest and dorsal neural tube precursors are present in a broad, crescent-shaped region of the gastrula. Surprisingly, static fate maps together with dynamic confocal imaging reveal that the neural plate border is considerably broader and extends more caudally than expected. Interestingly, we find that the position of the presumptive neural crest broadly correlates with the BMP4 expression domain from gastrula to neurula stages. Some degree of rostrocaudal patterning, albeit incomplete, is already evident in the gastrula. Time-lapse imaging studies show that the neural crest and dorsal neural tube precursors undergo choreographed movements that follow a spatiotemporal progression and include convergence and extension, reorientation, cell intermixing, and motility deep within the embryo. Through these rearrangement and reorganization movements, the neural crest and dorsal neural tube precursors become regionally segregated, coming to occupy predictable rostrocaudal positions along the embryonic axis. This regionalization occurs progressively and appears to be complete in the neurula by stage 7 at levels rostral to Hensen's node.

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Introduction

The neural crest cell is a highly motile cell type that arises within the forming central nervous system of vertebrate embryos. Presumptive neural crest cells reside within the elevating neural folds, which later appose to form the neural tube during neurulation. Around the time of neural tube closure, neural crest cells undergo an epithelial to mesenchymal transition, exit the dorsal neural tube, and, as individual cells, undergo extensive migrations into the periphery of the embryo. Following migration, they form diverse derivatives ranging from ganglia of the peripheral nervous system to pigment cells and the craniofacial skeleton.

In chick, the classical view of the neural crest has been that it is induced during neurulation by an inductive interaction between the neural and non-neural ectoderm as the neural folds elevate (Liem et al., 1995). This view is supported by experiments in which juxtaposing these two tissues recapitulates neural crest formation, even when taken from positions distant from the endogenous neural folds (Selleck and Bronner-Fraser, 1995). Moreover, BMPs and Wnts are capable of substituting for non-neural ectoderm in inducing neural crest cells from neural plate tissue (García-Castro et al., 2002). However, recent evidence suggests that presumptive neural crest in avian embryos is specified during gastrulation, before there is a clear neural and non-neural ectoderm (Basch et al., 2006). This early specification of the neural crest suggests that neural crest induction begins much earlier than previously thought and raises the intriguing question of where neural crest precursors first arise in the early embryo and how their position on the fate map relates to presumptive neural and epidermal tissues.

Although there have been several fate maps of the chick embryo generated at gastrula stages, none has carefully defined the origin of neural crest precursors, perhaps due to the fact that the neural crest was thought to form at a later stage. Here, we explore the origin of the neural plate border, the domain that contains both presumptive neural crest cells and dorsal neural tube precursors. Cell lineage analysis reveals that neural crest cells share a lineage with dorsal neural tube cells even after neural tube closure (Bronner-Fraser and Fraser, 1988), suggesting that neural crest cells do not become a fully segregated population until they delaminate from the neural tube. Previous fate maps of chick embryos at stages 3+ to 4 are somewhat variable, but show the future neural plate and neural crest crowning the node (Fernández-Garre et al., 2002; Bortier and Vakaet, 1992; Garcia-Martinez et al., 1993; Spratt, 1952; reviewed in Rodríguez-Gallardo et al., 2005).

In light of the finding that the neural crest is specified during gastrulation (Basch et al., 2006), it is important to re-address the early fate map, focusing on the position and extent of the neural plate

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border from which the neural crest derives. To this end, we have used a combination of dye labeling, careful positional analysis, and time-lapse imaging to fate map the precursors of the *n*eural *c*rest and *d*orsal *n*eural *t*ube (NC/dNT) at gastrula and early neurula stages. At gastrula stages, the results show that the neural plate fate maps further posteriorly than expected, and that the neural plate border is considerably broader than expected. As such, neural crest precursors arise adjacent to and interdigitated with neural plate derivatives in the stage 4 embryo. At the earliest stages, the rostrocaudal positions of NC/dNT cells are roughly predictive of their eventual positions along the neural axis; however, a few stages later (early neurula), the rostrocaudal position of NC/dNT cells strongly correlates with their later position, suggesting that the axial specification of the fate map is largely complete by mid-neurula stages.

To confirm the surprising aspects of the fate maps, we generated time-lapse sequences of individual, labeled embryos. These dynamic studies not only support the data obtained from the fate mapping experiments, but also reveal the dynamic and tightly choreographed behaviors that transform the gastrula fate map into the neurula fate map. Time-lapse sequences of cells at the neural plate border reveal that cells undergo extensive morphogenetic motions, including convergent extension, reorientation, cell mixing and dorsoventral motility. Thus, cell movements occur in all axes of the embryo. The results reveal the origin of the neural crest precursors and the movements that bring them to their position within the neural tube and explain the layout of the neural crest precursors in the static fate map.

Materials and methods

Embryos

Fertilized Colorado Reds and White Leghorn chicken eggs, obtained from local farms, were incubated at 38 °C for 18 to 24 h and 26 to 31 h to obtain stage 4 and stage 7 embryos, respectively (Hamburger and Hamilton, 1951). Intermediate incubation periods provided stage 5 and stage 6 embryos. Embryos were removed from the eggs using filter paper rings and cultured in thin egg white, according to a modified New culture method (Chapman et al., 2001). Briefly, eggs are opened and the thick albumin removed, reserving the thin albumin. A dry ring cut out of filter paper with a whole puncher is placed around the embryo and the embryo is cut out, following the edges of the filter paper ring. The embryos are gently rinsed in Ringer's saline, placed in 1 mL of thin egg white in a 35 mm tissue culture dish. Tissue culture dishes are arranged in a sealable plastic box lined with wet paper towels to maintain a high humidity environment. The plastic box is then placed in the incubator until the embryos reach stage 9 to 12 for embryos incubated at stage 4 and stages 13–15 for embryos incubated at stage 7. In later experiments, we found that the humidity of the tissue culture incubator was sufficient and the humidified plastic box was not needed.

Static fate mapping by DiI labeling

Fate maps of the neural crest were generated at stage 4 and 7 by placing a single injection spot of DiI on the dorsal surface of the epiblast of individual embryos. The mediolateral position of each spot was measured by determining its position relative to the primitive streak and the boundary between the area opaca and area pellucida (AO/AP). At stage 4, the rostrocaudal position of each spot *caudal* to the node was determined by measuring its position relative to the primitive streak (Fig. 1A); the rostrocaudal position of each spot *rostral* to the node was determined by measuring its position relative to an imaginary line that runs from the node to the anterior AO/AP boundary (Fig. 1B). The embryos were incubated overnight, until they reached stage 9 to stage 12, then they were fixed, embedded in gelatin and cryosectioned to identify those that contained staining in the dorsal neural tube and/or neural crest. The fate map was compiled by marking the coordinates of each individual injection spot onto the image of a representative stage 4 embryo, and color-coding the spots according to their fate at stage 9 to 12 (forebrain, midbrain, rostral or caudal hindbrain, trunk; Figs. 1C–F).

To create the fate map at stage 7, focal injections were made onto the forming neural folds, using the first formed somite (future somite # 2) both as a standard ruler and a landmark. We measured the distance of each spot in somite lengths rostral or caudal to the first somite. The embryos were incubated overnight, until they reached stage 12 to stage 15, then fixed, embedded in gelatin and cryosectioned to confirm that the embryos contained staining in the dorsal neural tube and/or neural crest. The fate map at stage 7 was compiled by plotting and color-coding each spot onto a representative stage 7 embryo (Fig. 8A).

Lipophilic dye labeling for cell movement analyses

To confirm the stage 4 and stage 7 static fate maps in individual embryos, we followed the movements of presumptive neural plate, neural crest and non-neural ectoderm cells by time-lapse analysis. Cells in embryos (stages 4–7) were labeled using alternating focal injections of DiI and DiO in a checkerboard pattern (see Figs. 3A, 7A, B and 9A, B). Since the cell populations were alternately labeled in green or in red, the time-lapse sequences revealed how different sets of cells migrate, mix and become localized to rostrocaudal and mediolateral positions along the embryonic axis. The alternating pattern also simplified the identification of the descendants after the embryos were fixed, embedded in gelatin and cryosectioned.

Time-lapse imaging method

Time-lapse sequences of embryos ranging from stage 4 to stage 10 were used to follow the motions of the DiI and/or DiO labeled cells. An imaging culture chamber was made from six-well dishes, from which we cut a hole in the bottom of one of the wells and sealed a number 1 cover slip in place. The embryo was placed, ventral side up, in 0.75–1.0 mL of egg white in the well with a cover slip. The remaining wells were filled with autoclaved double-distilled water to maintain high humidity in the chamber. An air bubbler within the incubator, filled with double-distilled water was connected to a compressed air tank (5% CO_2, 20% O_2, 75% N_2), and used to feed warmed, humidified, carbonated air into the chamber. The imaging chamber was placed on the stage of confocal microscope, which was surrounded by a home-built incubator housing, made from cardboard and insulated with reflectrix (Reflectix Inc, Markleville, Indiana). The warmth provided from an electric heating element was regulated with a temperature controller (Fisher scientific 11-463-47A) to maintain 37 °C (for more details, see Kulesa and Fraser, 2005).

Three-dimensional time-lapse sequences were generated on a Zeiss LSM-5 Pascal confocal laser-scanning microscope using the Pascal LSM software. Images were collected from the fluorescence channels and the transmitted light channel every 7 min for 12 to 18 h, with the laser power kept low (5 to 15%). The image sequences were processed for 3-D reconstruction using the *z*-projector plugin for ImageJ, version 1.32. Re-alignment of time-lapse sequences in which there was any shift in the specimen was performed using the TurboReg plugin for ImageJ.

Immunocytochemistry

Specimens were fixed in 4% paraformaldehyde for 24 h at 4 °C, and permeabilized in antibody buffer (Phosphate Buffered Saline (PBS)