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.

Keywords

Neural crest; dorsal neural tube; neural plate border; neural plate; cell movements; gastrulation; neurulation; chick; convergent extension; reorientation; deep motility; morphogenesis; fate map

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 stage 3+ to 4 are somewhat variable, but show the future neural plate and neural crest crowning the node (Fernandez-Garre et al., 2002; Bortier and Vakaet, 1992; Garcia-Martinez et al., 1993; Spratt, 1952; reviewed in Rodriguez-Gallardo et al., 2005).

In light of the finding that the neural crest is specified during gastrulation (Busch et al., 2006), it is important to re-address the early fate map, focusing on the position and extent of the neural plate 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 neural crest and dorsal neural tube (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 hours and 26 to 31 hours 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, preserving 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 stage 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 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; Fig. 1 C-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 Fig. 3A, Fig. 7A,B and Fig. 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\textsubscript{2}, 20% O\textsubscript{2}, 75% N\textsubscript{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 minutes for 12 to 18 hours, 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 hours at 4°C, and permeabilized in antibody buffer (Phosphate Buffered Saline (PBS) with 0.1% Tween, 5% goat serum and 2% albumin) for at least one hour before the primary antibody was applied at dilutions ranging from 1:1 to 1:500. At least 8 washes were done in large volumes of PBS with Tween before blocking with antibody buffer and applying the alexa-488 secondary antibody (1:200 dilution). After another 8 washes, the specimens were stained with 4′,6-diamidino-2-phenylindole (DAPI) to detect cell nuclei, mounted in permajfluor, and imaged on a Zeiss fluorescence microscope.

**Chromogenic in situ hybridization**

Embryos were fixed in 4% paraformaldehyde (PFA) and processed for in situ hybridization with the Bone Morphogenic Protein 4 (BMP4) probe. The antisense RNA probe, which was digoxigenin-11 UTP-labeled, was synthesized according to standard procedures (Wilkinson, 1992). Whole-mount in situ hybridization was performed by a combination of methods used for chick embryos (Wilkinson, 1992 and Henrique et al., 1995). Embryos were not incubated in proteinase K, and hybridization was carried out at 70°C. After completion of the in situ procedure, the embryos were post-fixed in 4% PFA at room temperature for 30 min., dehydrated in methanol and stored at −20°C.

**Double fluorescent in situ hybridization**

The double fluorescence in situ hybridization was a combination of the protocols of Denkers and colleagues (2004) and Acloque and colleagues (2008). Briefly, the BMP4 RNA probe was fluorescein UTP-labeled and the Pax7 probe was digoxigenin-11 UTP-labeled. Hybridization was carried out at 70°C, 5–6 post hybridization washes were done at 70°C over 36 hours. At least 10 post-hybridization washes were done in sterile TNT antibody buffer (0.1 M Tris pH 7.5, 0.15 M NaCl, 0.5% Tween20) over 24 hours, including one overnight wash at 4°C. Embryos were incubated in TNT containing 20% sheep serum and 2% Blocking Reagent (Roche Molecular Biochemicals; 11096176001) before the antibody incubation. We used the HRP-coupled anti-digoxigenin antibody and the HRP-coupled anti-fluorescein antibody at a dilution of 1:1000 and 1:500, respectively, in antibody buffer. Embryos were stained sequentially, first using the weaker probe (Pax7), which was developed in red by incubating in the fluorophore-tyramide working mix at a concentration of 1:100 in amplification diluent overnight at 4°C. The specimen was washed 10 to 15 times over at least 24 hours at room temperature. Inactivation was done in 1% hydrogen peroxide in TNT for 45 min to an hour.
before proceeding to the detection of the second probe (BMP4 probe), which was developed in green. Embryos were kept in PBS with 0.1% sodium azide at 4°C for long-term storage.

**Morphometric analyses**

**Extension of polyclones**—To determine the progressive increase in length of individual polyclones between stages 4 to 9, we measured the rostrocaudal length of each polyclone as the embryos developed from stage 4 to stage 9 in time-lapse sequences, using the measurement option in the Zeiss LSM software. We generated plots using Microsoft Excel 2004 for Mac. The percent change in the length of individual polyclones was determined by dividing the length at a given stage by the length at the previous stage, and multiplying by 100: \((\text{Length at stage } x / \text{Length at stage } x-1) \times 100\).

**Reorientation of clones**—We measured the change in orientation between pairs of individual dye spots from stages 4 to 8+/9 using ImageJ. Defining the mediolateral axis as 0°, the angle between pairs of spots that were originally mediolateral neighbors was measured at various stages. The resulting angles were plotted using Excel.

**Rostrocaudal correlation of the fate maps at stage 4 and stage 7**—An analysis of the correlation of the original and final positions of cells along the rostrocaudal axis was performed by scatter plotting the original and final positions of the dye-labeled clones against one another. For stage 4, we identified the rostrocaudal position of individual injection spots by measuring the rostrocaudal distance between the injection spot and the rostral boundary between area opaca and area pellucida. For stage 7, we measured the rostrocaudal distance between injection spot and the head fold. After a 24-hour incubation of the labeled embryos, each injection spot had spread along the embryonic axis, so the positions of the labeled descendants were plotted in each of the neural tube segment(s) to which they contributed. As a result, spots that labeled multiple segments are represented multiple times. Measurements were normalized and plotted on a representative stage 11 embryo outline.

The correlation factor \(r^2\) for each graph was calculated using Excel. A correlation factor of 0 would indicate a complete lack of correlation between the beginning positions and end point positions; a correlation factor of 1 would indicate a total correlation in which the beginning position predicts the final position perfectly.

**RESULTS**

**Fate map of the neural crest/dorsal neural tube at stage 4**

To map the positions of precursors of the neural crest and dorsal neural tube cells, chick embryos were labeled at various axial levels by focal injections of the vital dye, DiI, each resulting in labeling 50–200 cells. Embryos were analyzed after they had developed to stage 9–13, and the descendants of the labeled cells were scored for both their rostrocaudal position and their contribution to embryonic structures. A fate map was created by combining the results from multiple embryos onto the image of a standard stage 4 embryo (scaled as shown in Fig. 1A,B), color-coding the position of each dye-injection with the position and fate of its labeled descendants (Figure 1C–F). The fate map reveals that the cells contributing to the neural crest and dorsal neural tube (NC/dNT) are distributed in a broad crescent-shaped swathe that runs from near the top of the epiblast to approximately 1/3 the length of the epiblast; these cells partially overlap with the cells contributing to the more ventral neural tube (Fig. 1C,E). The stage 4 fate map shows a crude rostrocaudal ordering of the NC/dNT precursors (Fig. 1D,F). Thus the neural crest and neural plate arise from somewhat intermixed populations, with NC/dNT precursors enriched near the lateral edge of the neural plate. Mediolaterally, the crescent of NC/dNT precursors is surprisingly broad, spanning nearly half the distance from the midline.
to the area opaca. Figure 2 (A, B, D, E, G, H) shows three representative cases. The scoring of the cells as NC/dNT was confirmed in some embryos by immunostaining with an antibody to Pax7, which is expressed by cells in the dorsal neural tube (including the pre-migratory neural crest) and the migrating neural crest (Fig. 2 C, F and I series). The antibody also labels cells in the somitic mesoderm, but this does not confuse the analysis.

The static stage 4 fate map (Fig 1) shows a broad NC/dNT precursor domain that is transformed a few stages later to the longer and narrower neural fold. This transformation is reflected in the change in the shape of dye-labeled polyclones, from the initial round dot of 30–70 µm in diameter to an elongated shape of at least 200–400 µm along the rostrocaudal axis by stage 10–12. In the face of this dramatic narrowing and lengthening of the labeled polyclones, it is not surprising that there is only crude order to the stage 4 fate map. Note that the progenitors for forebrain (yellow) cluster to the rostral half of the fate map, while progenitors for the trunk (black) cluster more caudally in the fate map (Fig. 1D and F). The correlation of the position of the labeled dye injections and the positions of the labeled descendants will be analyzed in more detail below.

**Dynamic imaging confirms the stage 4 fate map**

The fate map of the stage 4 embryo (Fig. 1) suggests that the neural plate border is broad and has moderate rostrocaudal order. However, any static fate map combines data from several embryos, such that variation in the embryo stage or imprecision in the alignment of the data could make individual domains within the fate map appear artificially enlarged. To validate the apparent broadness of the NC/dNT domain in the stage 4 fate map, we employed intravitral imaging to determine the fates of multiple labeled spots in the same embryo. Alternating focal injections of DiI (red) or DiO (green) were made in a checkerboard pattern across the NC/dNT domain of the fate map within a single specimen (Fig. 3A; Suppl. Movie 1) and followed by laser scanning confocal microscopy until stage 9. Selected frames from a time-lapse series (Fig. 3A) shows that multiple spots can be followed over time so that the fate of each labeled spots can be scored at the end of the series (Fig. 3B). In this example, polyclones number 3, 4, 5, 8, 9 and 11 all contributed to the NC/dNT in the stage 9 embryo. To validate the scoring of the fates of the labeled clones, some embryos were labeled with only one DiI and one DiO injection, allowed to develop to stage 10–12, and scored after they were cryo-sectioned (n=17; Suppl. Fig.1). Injections separated by about 150 µm mediolaterally both contributed to the NC/dNT domain either as intermixed populations or as rostrocaudal neighbors. Thus, the large expanse of the NC/dNT domain in the static fate map is correct.

**Dynamic imaging shows the morphogenesis of the fate map at stage 4**

In the stages considered here, the originally flat embryonic blastodisc is transformed into the more 3-dimensional neurula. The region fated to become the neural plate border begins as a broad arc-shaped domain about halfway between Hensen’s node and the boundary between the area opaca and area pellucida. As the neural folds become evident at stage 6, this region narrows and elevates to form the neural folds, which then appose and fuse to form the neural tube. In this dramatic rearrangement, cells that were mediolaterally separated by more the 150 µm become near neighbors, and cells that were rostrocaudally quite close must move apart to form the elongated neuraxis.

To better understand this process, we followed the changes in the shape of the labeled polyclones (Fig. 4A,B) and the tracks of these clones (Fig. 4C) from stage 4 to stage 8 (Suppl. Movie 2). The labeled cells that contribute to the neural folds (polyclones 4,5,6,8 and 9; confirmed in cryosections) show the expected elongation in cranial regions (Fig. 4B, polyclones 4,5,6) and profound elongation in trunk regions (Fig. 4B, polyclones 8, 9). Tracking the motion of the rostral edge of the polyclones (Fig. 4C) reveals rearrangements predicted

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from our static fate mapping, with the cells following arched paths that end in the midline at different positions along the rostrocaudal axis. This suggests significant convergence motions, extension motions, reorientation and intermixing, which we study in detail in the following.

**Extension**—The extension along the rostrocaudal axis (Fig. 4A,B) can be followed easily by marking the rostral border of each labeled polyclone, and joining the marks by line segments (Fig. 4D). The resulting polygons show that there is extension. As the head process begins to extend forward from the node (stage 4+/5), polyclones contributing to the rostral neural tube (polyclones 4, 5, 6) move rostral to the primitive streak in concert with the formation of the head process. Extension progresses from rostral to caudal. This progression can be appreciated by following changes in the length of NC/dNT polyclones in the individual frames from the time-lapse sequence (Fig. 4E). The length of rostral polyclones increases before that of more caudal ones. In addition, extension of the polyclones progresses faster than the node advances caudally. Figure 4F shows that polyclones 8 and 9, both caudal to the node, are already extended.

The extension of the polyclones reveals that the rostrocaudal ordering of the stage 4 fate map is somewhat crude, as suggested in the static fate map (Fig. 1). Anterior polyclones tend to contribute to the most rostral portions of the dorsal neural tube, but there is overlap between polyclones contributing to different rostrocaudal levels. For example, in Figure 4, both polyclones 4 and 5 label the forebrain, but the caudal 1/3 of polyclone 5 extends into the midbrain; polyclone 6 also labels the midbrain. The rostrocaudal order can be seen clearly by comparing the position of rostral polyclones (4,5,6) to more caudal polyclones (8,9).

Analysis of the extension of polyclones in the dorsal neural tube shows that extension is more limited in magnitude at cranial levels (up to 6-fold) than at trunk levels (up to 25-fold; Fig. 5A,B). Despite these differences in magnitude, the extension movements are most rapid before stage 6 at both cranial and trunk levels (Fig. 5C,D). This timing correlates with the formation of the neural folds.

**Convergence**—In addition to extension, polyclones move from lateral to medial, converging toward the primitive streak. This movement occurs at both cranial and trunk levels, albeit more slowly at presumptive cranial levels. This difference is clear in Fig. 4D, where polyclones 10 and 7 reach the midline before polyclone 4. Within the neural plate border, there appears to be a lateral to medial bias, with polyclones 6 and 9 moving medially before polyclones 5 and 8. After stage 6, neural folds form, elevate and appose in a rostral to caudal fashion. After stage 6 in the head, medial displacement of the polyclones towards the midline is due to apposition of the neural folds.

**Reorientation**—With development, polyclones originally aligned mediolaterally relative to one another move to different rostrocaudal levels along the rostrocaudal axis. This movement involves a 90° reorientation of the clones relative to one another. For example, in Fig 4D, polyclones 4, 5 and 6, which are mediolaterally adjacent, move so that polyclone 4 (rostral forebrain level) becomes positioned rostral to polyclone 5 (caudal forebrain & rostral midbrain level), which is in turn rostral to polyclone 6 (midbrain level). Similar 90° reorientations are observed in the embryo in Figure 3A, where polyclones 3, 4, 5 (in the fore- and mid-brain) and polyclones 8, 9 (in the caudal midbrain and rostral hindbrain) are mediolateral neighbors at stage 4 (Fig. 3A) but become rostrocaudal neighbors by stage 9 at the end of the time-lapse (Fig. 3B). This rotation can be quantified by measuring the relative angle of adjacent polyclones as a function of embryonic stage (Fig. 6). This reorientation is less profound at trunk levels as the clones extend to such great lengths that resulting intermixing makes the rotation motions less noticeable.
**Cell Intermixing**—Tracking the two-color, checkerboard pattern of labeling over time permits an analysis of mixing between polyclones. Lateral polyclones (e.g. green polyclone 5 in Fig. 4) converge towards medial polyclone neighbors (e.g. red polyclone 4) and eventually mix, exhibiting a zone of yellow overlap (Fig. 4B, black arrow). Polyclones 8 and 9 in the caudal hindbrain and trunk show even more intermingling (Fig. 4B trunk). Analysis of confocal microscopy optical sections (cf. Fig. 4B) and cryosections (cf. Suppl. Fig. 1) of differently colored polyclones confirms these overlaps.

**Deep movements**—Between stages 4 and 9, the embryo changes from a flat three germ-layered blastodisc to an elongated neurulating embryo. To follow the dorsoventral motions of the labeled polyclones, stacks of confocal microscopy optical sections were re-sectioned so that cross-sections could be visualized and, alternatively, embryos labeled at stage 4 were fixed at various stages and cryosectioned (Fig. 6B). In this example, the transformation of the mediolateral axis to the dorsoventral axis of the neural tube can be followed as midbrain polyclones increases in dorsal-ventral extent. At stage 7, the neural folds become apposed at the midline and the polyclone thickens with the neural fold. At stage 9, a midbrain polyclone widens as labeled cells spread outward due to the expansion of the midbrain and some of the cells migrate out to the periphery (Fig. 6, stage 9). Thus, the movements of the labeled cells that are deep in the embryo mirror the movements of the labeled cells more dorsal in the embryo.

Taken together, the above results show that cells in the presumptive neural crest/dorsal neural tube undergo extensive cell rearrangements between gastrulation and early neurulation. Time-lapse analyses reveal thickening and deepening of the neural plate border, convergence and extension movements, rotation of local cell relationships, and intermixing that refine an initially broad neural plate border region into a far more discrete and narrow neural fold. Importantly, the movies confirm the crude rostrocaudal patterning in the early embryo that becomes refined by convergence/extension movements that transform the nearly circular disc-shaped embryo into a narrow oblong neuraxis. These extensive motions challenge any strict rostrocaudal or mediolateral order, and therefore explain the moderate degree of rostrocaudal patterning seen in the fate map.

**Fate map and morphogenesis of stage 5 and 6 embryos**

The fate map generated from cells labeled at stage 4 as well as the time-lapse studies show a very broad domain of NC/dNT precursors as well as dramatic rearrangements and elongation of the labeled domains. To follow the refinement of the fate map, we performed DiI/DiO checkerboard labeling at stages 5 and 6, followed by time-lapse imaging as the embryos developed to stage 9 (Fig. 7).

Time-lapse imaging at stage 4+ or at stage 5 reveals many of the same morphogenetic motions documented from polyclones labeled at stage 4. During the time the neural plate is open, polyclones in the border region can be seen moving with respect to one another, attaining different positions along the rostrocaudal axis. Convergence movements continue, bringing polyclones closer to the embryonic midline. In contrast to polyclones labeled at stage 4, polyclones labeled at stage 5 show decreased extension at the level of the regressing node and prechordal plate (which appears at stage 4+; Lopez-Sanchez et al., 2005). Thus, stage 4+/5- polyclones fated to populate the forebrain and midbrain have a smaller rostrocaudal extent than stage 4 polyclones fated to populate the fore/midbrain (compare stage 4 polyclone #5 Fig. 4B to the stage 5 polyclone #3 in Fig. 7A). Caudal to the forming node, convergent extension of stage 5 polyclones are similar to stage 4 polyclones. In summary, although the neural plate border continues to undergo convergent extension from stage 4+ to 5+, polyclones initiated rostral to the node show decreased extension when compared to stage 4 polyclones.
Comparisons between the movements of polyclones labeled at stage 4 and stage 6 shows greater differences, suggesting that the major motions are somewhat completed by this stage. This agrees with the predictions of the quantitative analysis of the motions of stage 4 polyclones, showing the greatest rate of change takes place between stages 4 and 5 (Fig. 5). By stage 6, the neural folds become clearly evident in transmitted light microscopy as regions of greater cell density. Stage 6 polyclones disperse less along the rostrocaudal axis than their counterparts at stage 4, and show dramatically less reorientation. Since there is less extension and less reorientation, polyclones labeled at stage 6 remain more focal and overlap less than those created at stage 4 (Fig. 7B).

Comparison of the data from stages 4 and 6 shows that the neural plate border undergoes morphogenesis faster than the node regresses. Convergent extension movements in the neural plate border begin as early as stage 4, before node regression begins at stage 4+/5. By stage 6, as the neural plate border becomes visibly denser than the medial neural plate, the increased cell density of the neural folds extends more caudally than the regressing node (Fig. 4F, Fig. 7A 2h30min and 7B 0h00 and 3h00). Even polyclones in the most caudal regions of the neural folds are undergoing convergent extension (Fig. 7B, polyclones #8 and #9). Thus, the wave of morphogenetic motions involved in the formation of the dorsal neural tube sweeps from rostral to caudal faster than the caudal-ward motion of the node.

**Fate map of the neural crest at stage 7**

Since the neural folds are clearly visible rostral to the Hensen’s node by stage 7 (1–2 somite stage), it should be possible to accurately and reproducibly distinguish presumptive NC/dNT from more ventral neural tube precursors. We find that focal dye injections targeted to the lateral region of the forming neural folds always label neural crest and/or dorsal neural tube. The dye-labeled polyclones from injections at stage 7 extend only approximately 100–200 µm along the rostrocaudal axis. This reliable rostrocaudal ordering extends from the level of the head folds to a few somite lengths caudal to the node (Fig. 8A). Further caudal, the domain of presumptive NC/dNT is broad and reminiscent of that seen at earlier stages. Reflecting the fact that gastrulation is ongoing caudal to the node, injections in this region give rise to varied cell fates including somitic mesoderm, neural plate, and extra-embryonic tissue.

The molecular identity of NC/dNT precursors was confirmed in selected embryos by immunostaining with an antibody to Pax7 (Fig. 8B–J). Descendants of the dye-labeled precursor cells were located within the Pax7 domain, in the dorsal aspect of the neural tube or along the migration pathway underneath the epidermis. Thus, DiI-labeled cells that contribute to the NC/dNT share the molecular markers indicative of cells fated to become dorsal neural tube and migrating neural crest cells.

**Morphogenetic movements of the neural plate at stage 7**

To understand how the presumptive neural crest cell populations move with respect to one another, we generated time-lapse sequences by labeling embryos with alternating focal injections of DiI and DiO into the neural folds of the presumptive midbrain, hindbrain and spinal cord. Although most injections were concentrated in the dorsal neural tube, a few were placed more medially in the neural tube.

Confirming the results of the static fate map, cell movements from stage 7 onwards are more orderly and less dramatic than seen at stage 4 (Suppl. Movie 3). At levels rostral to the node (future fore-, mid-, hindbrain), extension is reduced. The labeled polyclones remained round through the time-lapse sequence, showing little spreading along the rostrocaudal axis (Fig. 9A). Furthermore, cell rearrangement is limited. Polyclones labeled at stage 7 maintain their rostrocaudal position relative to their neighbors. For example, polyclones a through h retain...
their original rostrocaudal order through the end of the time-lapse sequence (Fig. 9B). The medial polyclones #1 and #2 also retain their medial position in the ventral neural plate, becoming covered over by the fusing neural folds. Convergence towards the midline at these later stages is a result of tissue folding, instead of cell movements. Figure 9C shows that polyclones initiated at stage 7 reside on the neural fold, and neural fold apposition brings this region to the midline for fusion (Fig. 9D).

**Comparisons between fate maps generated at different stages**

The fate maps and time-lapse sequences presented above suggest a progression from less ordered to more ordered over the few hours of development from stage 4 to stage 7. To permit a more direct comparison of the rearrangements seen in polyclones labeled at stages 4, 5, 6 and 7, Figure 10 presents time-lapse sequences from labeling performed at each stage. There is progressively less extension of polyclones established at later stages. The ratio of the lengths of individual polyclones drops from 5.4 for clones established at stage 4 (scored at stage 8+/9) to 1.2 for clones established at stage 7 (Suppl. Fig. 2).

To better define the degree of rostrocaudal patterning in the fate map of the neural plate border at stages 4 and 7, we analyzed the correlation between the locations of individual dye injection spots with their fates at stage 11. Figure 11 graphically depicts the fates for polyclones labeled at stage 4 or stage 7. The graphed fates for polyclones established at stage 4 shows dramatic scatter, resulting in a correlation coefficient of 0.4. In contrast, the graph for polyclones established at stage 7 is much more orderly, with a correlation coefficient of 0.9. Thus, over these few stages of development the fate map refines from something only loosely predictive of fate to a refined fate map.

**Relationship of BMP4 expression to the neural crest/dorsal neural tube fate map**

Bone Morphogenic Proteins (BMPs) are signaling molecules that have been implicated in the early steps in neural crest formation (Liem et al., 1995). At stage 4–5, we found that the BMP4 expression domain in chick correlates well with the broad neural plate border, which our fate map shows contains precursors for both the neural crest and dorsal neural tube (Fig. 12A; see also Streit and Stern, 1999). BMP4 is also expressed in tailbud regions caudally as well as in the presumptive epidermis. At stage 6, BMP4 was expressed in epidermis and neural folds in agreement with previous studies (Fig. 12B; Chapman et al., 2002; Basch et al., 2006; Otto et al., 2006; Sela-Donenfeld and Kalcheim, 2002). At stage 7, BMP4 is restricted to more caudal region (Fig. 12C). In addition, at stage 7, Pax7, a transcription factor that marks the dorsal neural tube, the migrating neural crest and the dorsal aspect of the somite, marks the entire rostrocaudal extent of the neural folds as previously described (Fig. 12D–F; Basch et al., 2006).

To determine if the BMP4 and Pax7 domains overlap at stage 7, we performed a series of double fluorescent in situ hybridizations. By this stage the expression of both markers has resolved to the neural folds, and confocal microscopy Z-stacks show that the BMP4 and Pax7 expression domains largely overlap; however, BMP4 expression extends more laterally than Pax7 at levels caudal to the first somite (Suppl. Fig. 3). In cryosections, Pax7 and BMP4 are co-expressed in the dorsal neural tube nuclei, especially more caudally (Fig. 12G–J). Rostrally, BMP4 expression is weaker in the neural folds. Pax7 expression extends medially to the sulcus limitans, while BMP4 is expressed laterally in the epidermis, with a few expressing cells in the more ventral neural tube/plate.

Superimposing the fate map of the stage 4 neural plate border on the expression pattern of BMP4 (Fig. 13), we find that the fate map falls within the BMP4 expression domain. The more
caudal region of the BMP4 expression domain abutting the primitive streak appears to label cells with non-neural fates.

**DISCUSSION**

Often called the fourth germ layer, the neural crest is an important cell population that gives rise to the peripheral nervous system among other derivatives. These cells form at the border of the neural plate (future central nervous system) and non-neural ectoderm (future epidermis) and serve as a useful model for studying the complex processes of induction and specification, migration and morphogenesis, differentiation and plasticity. Aspects of the chick neural crest fate map can be gleaned from fate maps focused on the neural plate (reviewed in Rodriguez-Gallardo et al., 2005). However, details of the position, organization and morphogenesis of prospective neural crest are not well known. To address this issue, we generated static and dynamic fate maps concentrating on the neural crest and dorsal neural tube, combining the techniques of lipophilic dye injection, *in vitro* embryo culture, and time-lapse imaging.

**The border region is wide at stage 4**

Our static fate map shows that the presumptive neural crest domain is surprisingly wide at stage 4 (Fig. 1). Mediolateral pairs of dye spots placed more than 100 µm apart in individual stage 4 embryos contribute to both the neural crest and/or dorsal neural tube (Suppl. Fig. 1). In addition, time-lapse sequences show that a relatively wide region contributes cells to the neural plate border and later to the dorsal neural tube/neural crest (Fig. 3 and Fig. 4). These experiments confirm that the neural plate border occupies a broad swathe that crowns the neural plate at stage 4, prior to forming the neural folds.

Previously published work, focusing on fate-mapping the neural plate, suggested that the neural plate border was approximately 35 µm wide (Fernandez-Garre et al., 2002). It seems likely that this difference results from the larger size of the labeled-graft used by Fernandez-Garre and colleagues, which may be more variable than our smaller injections spots. Our method of labeling polyclones by application of lipophilic dyes (Honig and Hume, 1989a,b) is less invasive than grafting and allows us to follow multiple dye spots in single embryos, thus offering a direct readout of the border’s width. Because the neural crest was not the main focus of their work, Fernandez-Garre and colleagues estimated, rather than directly assessed, the width of the border region.

The wide presumptive neural crest region in chick correlates with the broad expression domains of some genes in the border region at gastrulation and early neurulation stages. For example, BMP4 is expressed in a broad domain around the neural plate at stages 4 and 4+/5; Pax7 is located in the early forming folds by stage 6 or earlier (Chapman et al., 2002; Basch et al., 2006; Otto et al., 2006; Monsoro-Burq and LeDouarin, 2001).

**Dynamic cell behaviors drive neural plate border morphogenesis**

The neural plate border undergoes substantial transformation during morphogenesis, changing from a short, wide and flat sheet of cells to two long, narrow and 3-dimensional neural folds that appose and fuse rostrocaudally along the embryonic midline (van Straaten et al., 1997). Our time-lapse analyses show tightly choreographed convergent extension movements (lengthening and narrowing of a tissue) at the neural plate border (Keller et al., 2000; Schoenwolf and Alvarez, 1989; Lawson et al., 2001; Sausedo et al., 1997). In addition, regions of overlapping red-labeled and green-labeled cells show that cell intermixing occurs. This cell intermixing is probably mediated by mediolateral cell intercalation (or cell mixing along the mediolateral axis), which is known to drive convergent extension movements. Mediolateral cell intercalation has been observed by time-lapse analysis in fish, frogs and polychaete worms.
(cf. Warga and Kimmel, 1990; Steinmetz et al., 2007; Gong et al., 2004; Megason and Fraser, 2003). Our data from time-lapse sequences from stage 4 or 7 to stage 8+/9 show that the percent extension peaks between stages 4 and 5 and, although extension continues, it is slower thereafter (Fig. 5C,D and Suppl. Fig. 2).

The reorientation of mediolateral neighboring polyclones by 90° is an intriguing movement that is more pronounced in the head than the caudal hindbrain and trunk. During reorientation, mediolateral polyclones rotate with respect to one another and eventually become rostrocaudal neighbors. The net result is an increase in length and narrowing in width of the cranial neural folds. Lengthening of the polyclones along the rostrocaudal axis further increases the length of the head. While reorientation is minimal in the trunk, more dramatic lengthening of polyclones (up to 25-fold compared to 6-fold in the head; Fig. 5A and B) occurs in the trunk between stage 4 and 9. This difference in morphogenesis between head and trunk gives a striking image of mediolateral (horizontal) red and green (DiI/DiO) stripes, in the head while the hindbrain and trunk have rostrocaudal (vertical) stripes (Fig. 3B).

As cell populations converge, extend, reorient and intermix, they also change within the dorsoventral axis (Z-axis or depth of the embryo). The flatness and relative transparency of the early chick embryo permits the clear observation of cell behaviors along the dorsoventral, mediolateral and rostrocaudal axes. Following populations of labeled neural plate border cells shows that these populations increase in height (Fig. 6B stage 4–7). Next, labeled polyclones spread away from the surface of the epiblast, and dive deep into the embryo as the neural folds form, appose and are covered by non-neural ectoderm (Fig. 6B stage 9). Lastly, we see that the labeled populations begin to broaden mediolaterally as a result of growth of the forebrain, midbrain and rhombomeres (Fig. 6B stage 9). We followed the movements of polyclones at various depths along the Z-axis using confocal microscopy, and found them to be similar to those documented more superficially.

In summary, cell movements occur in all three orthogonal planes at the neural plate border and transform the flat and broad neural plate border into two elongated, narrow and apposed neural folds. Convergent extension brings the presumptive NC/dNT cells toward the midline and stretches the rostrocaudal axis. Reorientation positions cranial neural fold precursors appropriately along the rostrocaudal axis and also narrows the head. Displacement along the Z-axis thickens the forming folds, and brings them underneath the ectoderm. Convergence of the elevated folds towards the midline brings the mature folds together so that they can fuse along the dorsal apex, from which neural crest will emigrate. Thus, dynamic and varied cell movements drive the morphogenesis of the neural plate border during gastrulation and early neurulation.

Additional mechanisms involved in the morphogenesis from flat neural plate border to neural folds

Cell division takes place throughout gastrulation and neurulation in the chick epiblast, similarly to zebrafish but unlike the deep neural plate (sensorial layer) of *Xenopus* (Smith and Schoenwolf, 1987; Sausedo et al., 1997; Gong et al., 2004; Elul et al., 1997). Importantly, those cell divisions are oriented along the rostrocaudal axis in chick. In fish, randomizing cell division decreases axis elongation, demonstrating the importance of oriented cell division to lengthening the embryo (Gong et al., 2004). Thus, oriented cell division may play a role in elongating the neural plate border and neural folds in chick as well.

Extension and retraction of cell protrusions indicate active migration and have been observed during embryonic axis elongation. Time-lapse sequences of cells expressing fluorescent protein in chick show extensive protrusive activity (data not shown) which may facilitate movements and in turn result in convergent extension and morphogenesis of the neural fold.
(Brodland, 2006), similar to how cells intercalate in the chick primitive streak (Voiculescu et al., 2007). In fish and frog, such protrusive activity is oriented along the mediolateral axis, in a bipolar or monopolar direction, and regulated by the planar cell polarity pathway (Topczewski et al., 2001; Davidson and Keller, 1999; Elul and Keller, 2000; Wallingford et al., 2000; Wallingford et al., 2002; Myers et al., 2002; Hong and Brewster, 2006; Skoglund et al., 2008).

**Cell rearrangements in the neural plate border are spatiotemporally regulated and gradually segregate neural plate border cells**

Our in vivo time-lapse studies of differently labeled polyclones in the neural plate border reveal a spatiotemporal progression of convergence and of extension. Extension progresses in a rostral-to-caudal order. Rostral polyclones start spreading rostrocaudally (extending) earlier than their more caudal neighbors (Fig. 4E). Convergence of the neural plate border cells progresses with a lateral-to-medial bias. Following the distortion of gridded dye spots offers direct confirmation that lateral border cells begin their medial-ward (convergence) motion before medial border cells (Fig. 4D). This anterior-to-posterior progression of convergence and extension is reminiscent of that observed in *Xenopus*, where anterior and lateral neural plate cells are the first to develop a pointy, medial protrusion, to become motile and intercalate (Elul and Keller, 2000).

A gradual decrease in epiblastic cell rearrangements segregates dNT/NC cells into stable populations along the forming neural folds. Time-lapse series initiated at stage 4 show extensive polyclone movements. In fact, as a result of convergence, extension and reorientation: 1) labeled cells from a single polyclone disperse along the rostrocaudal axis, often labeling more than one brain segment or large stretches of the spinal cord by stage 10 (Fig. 3B, Fig. 4B and Fig. 10), and 2) medial and lateral polyclones mix through reorientation as well as mediolateral intercalation, giving rise to overlapping sections of dorsal neural tube (Fig. 4B, black arrows). By stage 5, cells rostral to the node begin to extend less along the rostrocaudal axis (Fig. 5C,D, Fig. 7A and Fig. 10). This trend continues through stage 7, by which stage cell rearrangements have decreased significantly. Dye spots on the stage 7 neural folds generally maintain their original shape through stage 9, extending little (Fig. 5C,D, Fig. 9 and Fig. 10 and Suppl. Fig. 2). Neural plate border cells are cleanly segregated, giving rise to distinct populations of neural crest cells along the rostrocaudal axis (Fig 11). As a result, the fate map at stage 7 shows clear segregation of color-coded polyclones along the rostrocaudal axis compared to the stage 4 fate map.

**Cell movements at various axial levels**

With a few key differences, the movements of presumptive neural crest cells at gastrulation and early neurulation (before delamination) are largely similar at all axial levels. First, both future head and trunk neural crest cells undergo extension in a rostral-to-caudal progression (Fig. 4E). Secondly, convergence follows a rostral-to-caudal progression with a lateral-to-medial bias in head and trunk (Fig. 4D). Thirdly, morphogenesis of the neural plate border precedes regression of the node in both locations (Fig. 3A, Fig. 4F and Fig. 7A,B). Finally, oriented cell division occurs equally in both head and trunk domains (Smith and Schoenwolf, 1987; Sausedo et al., 1997). An important difference between presumptive head and trunk is that the neural plate at gastrula stages is much broader at the head level than at the trunk level, as is true across phyla (frog: Keller, 1976; Eagleson and Harris, 1990; fish: Woo and Fraser, 1995; Myers et al., 2002; worm: Steinmetz et al., 2007; chick: Rodríguez-Gallardo et al., 2005). In the neural plate border in chick, reorientation occurs mainly in the head in a lateral-to-medial progression. Other mechanisms that could maintain a wider head domain include fewer rounds of mediolateral intercalation or more proliferation. Different cell rearrangement patterns have been seen in the head and the trunk in other species; for example, cells in the head of worms...
undergo convergence but not extension, and those in the mouse head exhibit uniform cell growth across the head domain compared to the trunk (Mathis and Nicolas, 2000; Steinmetz et al., 2007).

Induction and specification of the neural plate border region

A subset of chick neural crest cells is specified early (stage 3–4; Basch et al., 2006). These neural crest cells reside in a domain approximately 80 µm wide, and located “perpendicular to, and 250 µm caudal to the most rostral extent of the primitive streak of stage 3–4 embryos.” A closer look at their figure 2B suggests that this prospective neural crest domain fate maps to the dorsal neural tube at the level of rostral hindbrain or midbrain. At stage 3–4, BMP4, but not yet Pax7, is expressed in the border region (Fig. 12). Therefore, we deduce that it is the rostral hindbrain or midbrain neural crest cells that are specified early, and that they express BMP4 early and Pax7 slightly later. There are cases where presumptive neural crest are located far laterally, away from invaginated mesoderm; these may comprise a neural crest population that is specified in the absence of mesoderm (Basch et al., 2006). This precedes the classical model of neural crest induction, which involves interaction between the neural plate, epidermis and/or mesoderm at open neural plate stages (reviewed in Meulemans and Bronner-Fraser, 2005; Basch et al., 2004, in Aybar and Mayor, 2002 and in Raible and Ragland, 2005; Selleck and Bronner-Fraser, 1995; Marchant et al., 1998; Bonstein et al., 1998).

In summary, we have fate mapped the presumptive neural crest and dorsal neural tube both statically and dynamically. The results show that these precursors occupy a wide border that overlaps with BMP4 at stage 4; through narrowing, lengthening, reorientation, cell intermixing, and dorsoventral cell displacement, the future neural crest cells become restricted to and segregated along the neural folds at stage 7 and the dorsal neural tube at stage 10, overlapping with expression of Pax7.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Fate map of the neural crest and the dorsal neural tube

(A, B) Method for plotting position of polyclones on the fate map. (A) For injections located caudal to the node, the position of the dye-labeled polyclones was placed on a standardized stage 4 embryo by determining for each embryo the position of the 30–70 µm spot of DiI relative to the height of the primitive streak and to the distance between the center of the streak and the AO/AP (Area Opaca/Area Pellucida) boundary at that particular rostrocaudal level.

(B) For injections located rostral to the node, the mediolateral position was determined relative to an imaginary line between the area opaca and primitive streak; rostrocaudal position was determined relative to a line from Hensen’s node to the apex of the embryo. Plotting the positions using these percentages automatically compensates for variations in the size and
shape of individual embryos. See Materials and Methods for additional details. (C) Color-coded fates of polyclones labeled at the depicted positions. Note that the neural crest and neural plate overlap at stage 4. For clarity, data from only a subset of the polyclones are depicted. In total, over 130 embryos were sectioned to validate the cellular identity of the labeled cells, 50 had perfect morphology and were used to define the fate map. (D) Color coded fate map of the neural crest showing rostrocaudal identity of the polyclones. Multiply colored spots show polyclones that contributed to more than one axial level. (E) Close-up of panel (C). (F) Close-up of panel (D).
Figure 2. The neural plate border region at stage 4 contributes to neural crest
Three representative embryos labeled with DiI at stage 4 in the neural plate border region of
the fate map, cryosectioned and stained for Pax7, a marker of the dorsal neural tube, migrating
neural crest and dorsal somite. Three different axial levels of injections into the neural plate
border are presented:
A–C” shows an embryo labeled in the neural plate border at the rostral hindbrain level; D–
F” in the neural plate border at the level of the otic vesicle; G–I” in the neural plate border at
the caudal hindbrain level.
The “t=0” column shows images of the embryos at the time of injection; the “t=24hrs” column
shows the same embryos after one day and the right three columns show sections through the
labeled regions of those same embryos.
Scale bars: 200 µm, except C–C”, F–F”, I–I” 50 µm.
Figure 3. Frames from a time-lapse sequence from stage 4 to stage 9

(A) Polyclones change shape and position. Note the change in individual polyclones—for example #4 and #14—between the beginning and the end of the time-lapse sequence. Unnumbered polyclones gave rise to extra-embryonic membrane, polyclones #6 and 7 labeled medial neural plate and became covered over by the fusing neural folds, #13 moved caudally and out of view on the computer screen during development. (B) Collage of images from z-stacks generated from this embryo at the end of the time-lapse, showing the different patterns of polyclones in the head versus trunk.
Figure 4. Analyses of convergent extension at the neural plate border

(A) Beginning frame from the time-lapse sequence of the embryo analyzed throughout this figure. The polyclones are numbered. (B) Image from a confocal z-stack generated at the end of the time-lapse sequence (embryonic stage 8+). Black arrows point to regions of mixed red and green cells. Note also how polyclones occupy different axial levels, testifying to the occurrence of reorientation. (C) Cell tracks of those polyclones, with the solid circle indicating the beginning of the cell path and the red tip indicating the end of the path. The tracks shown in black represent polyclones that did not give rise to neural fold. The green, peach and mauve tracks originate in the neural plate border and give rise to neural folds. P.S.: Primitive Streak. (D) To show the cell movements of the neural plate border and give rise to neural folds, the polyclones were rendered as vertices of a grid. As each polyclone moved, the displacement of the corresponding vertex was followed to determine the distortion of the neural plate border, due to 1) extension, 2) reorientation, and 3) convergence towards the midline.
(E) Frames from the time-lapse sequence of the embryo shown in (A), showing that extension progresses rostral to caudal. Polyclones are numbered at t=0hrs, and followed over time. Numbered asterisks show polyclones beginning extension and asterisks without numbers show those continuing extension. Note the rostral-to-caudal progression as polyclones #6 and #5 start extending before polyclones #9 and #8.

(F) Frame from the same time-lapse as in (A) showing that formation of the neural folds progresses faster than the node regresses. The caudal extent of the forming neural folds lies caudal to the level of regressing node. Polyclones #9 and #8, both on the fold and both caudal to the node, have already extended considerably. CaudNF: caudal extent of neural fold. Frame at stage 6, 10h23min within the time-lapse sequence.

Scale bars: 200 µm. P.S.: Primitive Streak; P.P.: Prechordal plate; Ntc: notochord; Hf: Head fold; Fb: forebrain; Mb: Midbrain; rHb: Rostral Hindbrain; cHb: caudal Hindbrain (level of somite 1 or 3 as specified).
Figure 5. Quantitative analysis of polyclone length and percent increase in length

(A, B) Polyclone lengths were measured at each stage, and show an increase in length over time in both the head (A) and in the hindbrain and trunk (B). (C, D) Although growth continues, the percent change in polyclone length shows a peak of extension occurs between stages 4 and 5.
Figure 6. Reorientation and deep movements
(A) In the cranial regions of the embryo, rows of polyclones reorient along the rostrocaudal axis. The angle (0° defined as the mediolateral axis) between originally mediolateral neighboring polyclone pairs at the beginning of the time-lapse (stage 4), the middle (stage 6) and the end point (stage 9) increases with development. (B) Representative polyclones initiated at stage 4 on different embryos, which were fixed at various stages. At stage 7, the polyclone labels the rising neural fold and the height of the labeled cell population has increased. At stage 9, the polyclone spreads both deep into the embryo and laterally, due to growth of the brain vesicle.
Figure 7. Time-lapse of Dil/Dio polyclones labeled at stage 5 and stage 6
(A) Time-lapse frames show that dye injection spots made at stage 5 undergo less rostrocaudal extension than spots made at stage 4 (e.g. Fig 3). (B) Time-lapse frames of dye injection spots made at stage 6 showing an even greater reduction in rostrocaudal extension than observed at earlier stages. CaudNF: caudal extent of neural fold. Scale bars: 200 µm.
Figure 8. Fate map of the neural plate border region at stage 7

(A) Fate map of the stage 7 embryo shows that the presumptive neural crest is well ordered along the rostrocaudal axis. The color code is indicated below the fate map. (B–J) Three representative embryos labeled at different axial levels with DiI at stage 7 in the neural fold, cryosectioned and stained for Pax7. In all three cases, the DiI injection spot labeled neural crest and dorsal neural tube cell populations. 

B–D” An embryo labeled in the neural plate border at the trunk level; E–G” An embryo labeled in the neural plate border at the level of the otic vesicle; H–J” An embryo labeled in the neural plate border at the caudal hindbrain level. 

The “t=0” column shows images of the embryos at the time of injection; the “t=18hrs” column shows the same embryos after one day and the right three columns show sections through the labeled regions of those same embryos.

Scale bars: 200 µm, except D-D”, G-G”, J-J” 50 µm.
Figure 9. Reduced rostrocaudal rearrangements of polyclones labeled at stage 7

(A) Frames from a time-lapse sequence started at stage 7. The dye spots converge towards the midline as the neural folds come into apposition. These dye labeled polyclones do not elongate dramatically as the embryo develops, unlike polyclones labeled at earlier stages, denoting a decrease in extension after stage 7. (B) Polyclones (labeled with lower case letters) on the neural fold maintain their respective rostrocaudal position along the neural tube. This denotes an almost total absence of rostrocaudal reorientation along the axis. (C, D) Polyclones initiated at stage 7 are on the elevating neural fold (C). By stage 9, apposition and fusion of the folds brings the polyclones to the dorsal aspect of the neural tube. Scale bars: A and B 200 µm, C and D 50 µm.
Figure 10. Extension of the embryonic axis slows over time
The changes in the extension rate can be gauged by labeling different embryos at progressively older stages and generating time-lapse sequences to stage 9. Extension of polyclones marked at stage 4, 5, 6, or 7 and followed through stage 9 is decreased with the age of the embryo at labeling. Labels applied at stage 4 undergo the greatest amount of rostrocaudal extension; those applied at stage 7 show the least. The dye spots chosen to illustrate this point are each located in the caudal hindbrain/trunk area of the fate map (in the area of somite 1). Scale bar: 200 µm.
Figure 11. Correlation of positions at stage 4 and stage 7 with respect to stage 11
The position of individual injection spots at stages 4 or 7 is plotted along the y-axis. The position of the polyclone at the end of 24 hours re-incubation period is plotted along the x-axis. The correlation factor \((r^2)\) for each graph is presented to quantitatively assess the refinement of the fate map.
Figure 12. Pax7 and BMP4 expression overlap in the early embryo

(A, B, C) Stage 4, 6 and 7 embryos processed for mRNA in situ hybridization with a BMP4 probe. (D, E, F) A stage 7 embryo processed for double fluorescent in situ hybridization with BMP4 and Pax7 mRNA probes. The expression of transcripts overlaps in the neural folds. (G and I) Sections of embryos at the levels indicated in (D). (H, J) Higher magnification images of the boxed regions shown in G and I, respectively. Scale bars: 200 µm, except H, J 100 µm.
Figure 13. Fate map of the neural plate border superimposed on the expression of BMP4 at stage 4

The NC/dNT region of the fate map matches nicely with the expression of this gene in the neural plate border, indicating that presumptive neural crest cells arise in a region positive for BMP4. The fate map of the neural plate border shows an intermixing of rostrocaudal fates within the region of BMP4 expression. The large rostrocaudal expansion and mediolateral rearrangements of dye labeled polyclones from stage 4, and this intermixing of the fate map both support the hypothesis that presumptive neural crest cells have not acquired a definitive rostrocaudal identity at stage 4. Scale bar: 200 µm.