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Cellular basis of neuroepithelial bending during mouse spinal neural tube closure

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SUMMARY

Bending of the neural plate at paired dorsolateral hinge points (DLHPs) is required for neural tube closure in the spinal region of the mouse embryo. As a step towards understanding the morphogenetic mechanism of DLHP development, we examined variations in neural plate cellular architecture and proliferation during closure. Neuroepithelial cells within the median hinge point (MHP) contain nuclei that are mainly basally located and undergo relatively slow proliferation, with a 7 h cell cycle length. In contrast, cells in the dorsolateral neuroepithelium, including the DLHP, exhibit nuclei distributed throughout the apico-basal axis and undergo rapid proliferation, with a 4 h cell cycle length. As the neural folds elevate, cell numbers increase to a greater extent in the dorsolateral neural plate that contacts the surface ectoderm, compared with the more ventromedial neural plate where cells contact paraxial mesoderm and notochord. This marked increase in dorsolateral cell number cannot be accounted for solely on the basis of enhanced cell proliferation in this region. We hypothesised that neuroepithelial cells may translocate in a ventral-to-dorsal direction as DLHP formation occurs, and this was confirmed by vital cell labelling in cultured embryos. The translocation of cells into the neural fold, together with its more rapid cell proliferation, leads to an increase in cell density dorsolaterally compared with the more ventromedial neural plate. These findings suggest a model in which DLHP formation may proceed through 'buckling' of the neuroepithelium at a dorso-ventral boundary marked by a change in cell-packing density.

1. Introduction

Neurulation is the embryonic process by which the neuroepithelium bends and fuses dorsally to create the closed neural tube. A key morphogenetic component of neurulation is the stereotypical pattern of neuroepithelial bending by which the neural folds become elevated and apposed, enabling the adhesion and fusion that completes neural tube closure. In the spinal region of the mouse embryo, the neural plate bends acutely and focally at the midline (the median hinge point; MHP), creating the 'neural groove' with a V-shaped cross-section, and at paired dorsolateral hinge points (DLHPs) to generate longitudinal furrows that bring the neural fold tips towards each other in the dorsal midline. A switch from MHP to DLHP bending occurs as closure progresses rostro-caudally along the body axis (Shum and Copp, 1996; Ybot-Gonzalez and Copp, 1999). The *Zic2*-mutant mouse fails to develop DLHPs and subsequently exhibits extensive spina bifida (Elms et al., 2003; Ybot-Gonzalez et al., 2007), demonstrating the critical requirement for DLHP formation in the closure of the spinal neural tube.

The pattern of MHP and DLHP bending in the neural plate is regulated by mutually antagonistic dorsal and ventral embryonic signals. MHP bending is stimulated by notochordal factors including sonic hedgehog (Shh), whereas DLHP formation is simultaneously inhibited by Shh (Ybot-Gonzalez et al., 2002). Bone morphogenetic protein-2 (BMP2) secreted from the dorsal-most surface ectoderm also inhibits dorsolateral bending whereas Noggin, secreted from the tips of the neural folds, overcomes BMP2-mediated inhibition and enables DLHPs to form (Ybot-Gonzalez et al., 2007). In the upper spine, Shh is expressed strongly in the notochord and suppresses Noggin-mediated DLHP formation whereas, in the low spine, Shh production from the notochord is greatly diminished, Noggin is de-repressed, blocks BMP2 action, and DLHP formation occurs (Ybot-Gonzalez et al., 2007).

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Contraction of apical actin microfilaments is conventionally considered essential for neural plate bending. However, mice with targeted defects of cytoskeletal genes – while often exhibiting cranial neural tube defects – rarely show closure defects of the trunk region (Copp, 2005). Moreover, cytochalasins that disassemble actin microfilaments disrupt cranial neural tube closure, whereas the process of spinal neural tube closure is cytochalasin-resistant and both MHP and DLHPs are formed properly (Ybot-Gonzalez and Copp, 1999). Hence, other mechanisms may be responsible for the bending of the neural plate in the spinal region.

Neuroepithelial shape change is a key factor in MHP formation. The pseudostratified neuroepithelium exhibits interkinetic nuclear migration, between basal and apical locations as the cell cycle progresses (Sauer, 1935; Langman et al., 1967). In chick and mouse embryos, the MHP is populated mostly by wedge-shaped cells with basally located, mainly S-phase, nuclei. In contrast, non-bending regions of the neural plate comprise a mixture of cells with wedge, spindle and inverted-wedge shapes (Schoenwolf and Franks, 1984; Smith et al., 1994). Hence, bending of the neural plate at the midline may be attributed to the preponderance of wedge-shaped cells due to the basal location of the interphase nuclei, as the cells spend longer in S-phase (Smith and Schoenwolf, 1987; Smith and Schoenwolf, 1988).

During chick neurulation, the DLHP comprises 55% wedge-shaped cells, compared with more than 70% in the MHP and fewer than 35% in non-bending neural plate (Schoenwolf and Franks, 1984). Neurulation in the mouse has not been subjected to such detailed cellular analysis, and it is unclear whether DLHP bending in the mouse neural plate is accomplished by a similar cellular wedging as at the MHP. Here, we found that DLHPs do not differ from non-bending neural plate in their content of cells with basal nuclei, arguing for a different mechanism of DLHP formation compared with the MHP. Analysis of cell proliferation during DLHP formation, combined with neuroepithelial vital cell labelling, reveals a net ventral-to-dorsal translocation of cells within the plane of the elevating neuroepithelium. Cells congregate in the dorsal neural fold and differential cell packing densities result, dorso-ventrally, within the neuroepithelium. A physical discontinuity dorso-ventrally may lead to neural plate bending specifically at the DLHP. These findings highlight a novel mechanism of epithelial morphogenesis during closure of the mammalian neural tube.

2. Materials and Methods

2.1. Mouse strains and embryo preparation

Mouse procedures were performed under the auspices of the UK Animals (Scientific Procedures) Act 1986 and 'Responsibility in the Use of Animals for Medical Research' (Medical Research Council, 1993). Mouse strains were: inbred CBA/Ca for morphometric analysis; random-bred CD1 mice for surface ectoderm removal; random-bred ARC/s and inbred BALB/c mice for live cell labelling. Embryonic day (E) 0.5 was noon on the day after overnight mating. Embryos were dissected in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal calf serum (FCS), staged by counting somites and rinsed in phosphate buffered saline (PBS) prior to fixation. For ^{3}H-thymidine labelling, surface ectoderm removal, or live cell labelling, embryos were cultured whole, within yolk sac and amnion, as described (Cockroft, 1990). Surface ectoderm was removed surgically (Ybot-Gonzalez et al., 2002).

2.2. Morphometric analysis

Embryos were fixed for at least 24 h at 4 °C in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, washed twice in distilled water and post-fixed in 1% aqueous osmium tetroxide for 10 min at room temperature, followed by 1-2 h at 4 °C. After two 10 min washes in distilled water, embryos were dehydrated through an ethanol series, followed by two 10 min changes of propylene oxide, then resin/propylene oxide (1:1), before embedding in Epon resin (Agar). Semi-thin transverse sections, 2.5 μm thick, were prepared using a glass knife on a Philips ultramicrotome. Sections were stained with 1% toluidine blue/ 1% borax, and mounted in DPX.

Embryos within specific somite number ranges were selected for analysis, in order to represent the characteristic morphology of the three Modes of neurulation: Mode 1 (MHP only), 10-12 somites; Mode 2 (MHP and DLHPs), 19-21 somites; Mode 3 (DLHPs only), 28-30 somites. Five embryos of each Mode were analysed with data gathered in each embryo from two rostro-caudal levels of the posterior neuropore (PNP): flat and elevated neural plate (Fig. 1).

Nuclear position data were collected from three sections, spaced every 10 μm, within each of the flat and elevated neural plate levels. Since the diameter of neuroepithelial nuclei is approximately 8 μm, this spacing of the sections precluded repeat analysis of the same nucleus. Within each section, three specific areas were assessed: median hinge point (MHP), dorsolateral hinge points (DLHPs), and the straight lateral region (LAT), defined as indicated by the dotted lines in Fig. 2A and Fig. S1B-D. Five nuclei were scored per section in MHP and DLHP and eight per section in LAT. Sections were projected from a digital camera to a computer monitor, and the mid-point (apico-basally) of each nucleus was recorded as occurring in the apical, middle or basal thirds of the neuroepithelium (as shown in Fig. 2A). Proportional distribution of nuclei between the three apico-basal thirds formed the basis of the quantitative comparison of nuclear position.

Other morphometric comparisons were based on calibrated linear measurements and nuclear counts obtained from each half of three transverse sections, at flat and elevated PNP levels, in five embryos per Mode. Neuroepithelial width was measured along the basal surface, firstly along the neural fold, where adjacent to the surface ectoderm (nf in Fig. 1 K, L; Fig. 2E), and secondly along the remaining neural plate to the midline (npr in Fig. 1 K, L; Fig. 2E). Total neural plate width was the sum of nf and npr measurements. Data from replicate sections were pooled for each neural plate sub-division at each level of each embryo. Overall means±SEM were calculated for the 5 replicate embryos at each Mode. Cell numbers were determined by counting nuclei using the same neural plate sub-divisions as for width measurements. Nominal cell width was calculated by dividing mean neuroepithelial width by mean nuclear/cell number for the same region. Cell packing density was calculated by dividing the cell number of each neural plate region by its transverse sectional area, determined using the Measure/Analyse function of ImageJ.

2.3. Cell cycle analysis

In preliminary studies, bromodeoxyuridine was found to penetrate cultured embryos poorly, and so ^{3}H-thymidine was used for the cell cycle analysis. Embryos were cultured for 1 h from E9.5 and then ^{3}H-thymidine (25 Ci/mmol) was added to a final concentration of 1 μCi/ml. For the main analysis, embryos were harvested from culture at 0.5 h time intervals up to a maximum of 8 h. Only embryos with 18-22 somites (Mode 2 neurulation) after culture were included in the analysis of cell cycle kinetics. Autoradiography was performed on 2.5 μm-thick plastic transverse sections through the PNP, using Ilford K5 emulsion diluted 1:2 with 2% glycerol solution. Nuclei were considered labelled if the grain count exceeded background level, determined from an