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Defining a PARticular Pathway of Neural Tube Closure

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Mammalian neurulation is completed when the dorsolateral neural folds bend inwards, their tips make adhesive contacts across the midline, and the epithelia remodel to create a closed neural tube. Two recent papers (one by Camerer et al. in this issue of *Developmental Cell*) demonstrate a vital role for protease-activated G protein-coupled receptor signaling in these late closure events, opening up new avenues for exploring the molecular basis of mammalian neural tube morphogenesis.

Neural tube closure is the culminating event of neurulation, in which the neuroepithelium differentiates from nonneural ectoderm and then undergoes a series of morphogenetic events to create a closed tube. If the neural tube fails to close, the later events of neurogenesis and formation of nerve connections are interrupted by degeneration of the exposed neuroepithelium. Loss of brain neuroepithelium, as in anencephaly (failed cranial neural tube closure), prevents the neurological control of vital functions like respiration and is lethal. Degeneration of spinal neuroepithelium, as in open spina bifida, typically involves loss of neurological function below the level of the lesion, with paralysis and severe sensory deficit.

Despite the clinical importance of neural tube closure, we remain relatively naive in our understanding of its cellular and molecular regulation. One reason for this is the scientific myopia that has resulted from an excessive emphasis on the idea that apical constriction of actomyosin structures represents the primary mechanical basis for neurulation. Neuroepithelial cells certainly change shape as the neural plate bends and the neural folds elevate, but increasing evidence indicates that the driving forces of neurulation comprise more diverse events than simply apical microfilament contraction. For example, the sites of neural plate bending (median and dorsolateral hinge points) are neither strongly enriched for actomyosin nor particularly sensitive to drugs that disassemble microfilaments (Ybot-Gonzalez and Copp, 1999). We need to look more widely to gain a true impression of the range of cellular and molecular events that likely underlies the stereotypical bending, adhesion, fusion, and tissue remodeling events of mammalian neural tube closure.

Fertile ground for defining the cellular rules of neurulation is the panoply of mouse mutants and knockouts with neural tube defects (NTDs). While more than 150 mutants have this phenotype (Harris and Juriloff, 2007), only a few have been subjected to the sort of in-depth study necessary to implicate a particular cellular mechanism. In this issue of *Developmental Cell*, Camerer et al. (2010) begin to unravel a cascade of molecular interactions involving the protease-activated receptors (PARs) and their productive (i.e., signal-generating) cleavage by cell-associated tissue proteases. While nullizygosity for either PAR1 or PAR2 is compatible with normal neural tube closure, the double homozygote fails in cranial neurulation and exhibits mid/hindbrain exencephaly, the developmental forerunner of anencephaly. Multiple serine proteases can activate PAR1 and PAR2, with a number expressed in the neurulation stage embryo. Camerer et al. showed that PAR2, the predominant G protein-coupled receptor (GPCR) in this system, is cleaved and activated by the transmembrane protease matriptase, which itself is cleaved and activated by several other cell-associated proteases, including prostasin and hepsin. Strikingly, however, Szabo et al., in another recent paper, show that mice lacking the matriptase inhibitor HAI-2 also develop exencephaly and spina bifida (Szabo et al., 2009). Genetic deletion of matriptase was able to rescue these NTDs in a dose-dependent manner. Together, these papers show that overactivation is as damaging as underactivation, and that precise regulation of these proteases is essential for successful neurulation.

Since PARs are GPCRs, the next step was to test whether downstream G proteins are necessary for neural tube closure. Combined loss of $G\alpha_q$ and $G\alpha_{11}$ does not result in NTDs (Offermanns et al., 1998) and double mutants for $G\alpha_{12}$ and $G\alpha_{13}$ were also found to neurulate normally. However, elegant use of Cre-activated pertussis toxin expression (to inactivate $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, and $G\alpha_o$) together with Cre-mediated deletion of $G\alpha_z$ demonstrated that members of the $G_{i/o/z}$ family of GPCRs are indeed required for neural tube closure. Unlike the generalized PAR knockouts, the loss of $G_{i/o/z}$ family members was engineered using Cre-loxP technology to affect only the nonneural (surface) ectoderm. This followed the finding that PAR2 and probably also PAR1 are expressed at this location, on the outside of the neural folds (Figure 1A). Despite this highly specific targeting approach, NTDs still occurred, arguing for a localized requirement for GPCR function in nonneural ectoderm. Fascinatingly, however, the focus of the defects in the conditional $G_{i/o/z}$ deleted mice shifted from mainly exencephaly to predominantly spina bifida. Hence, cranial and caudal closure may have differing requirements for specific pathways of G_i-coupled GPCR signaling.

Likewise, when Camerer et al. examined requirements for *Rac1*, a GTPase regulated by GPCRs, a high frequency of both exencephaly and spina bifida was observed, suggesting that multiple GPCRs, whose importance for closure may vary with axial level, may converge

on Rac1 function in their downstream action. That said, the Cre-driver used in all the $G_{i/o/z}$ and Rac1 experiments deletes one allele of *grainyhead-like 3* (*Grhl3*), a gene already implicated in posterior neural tube closure. Interestingly, spina bifida in *Grhl3* mutant embryos results from a distinct requirement in the hindgut (Gustavsson et al., 2007). Therefore, it remains possible that heterozygosity for *Grhl3* caused by the Cre knockin may have interacted with loss of GPCR function in hindgut, rather than nonneural ectoderm, to promote spinal rather than cranial NTDs.

Rac1 is a known mediator of cytoskeletal reorganization, promoting cellular protrusions and cell motility, in contrast to the stabilization of cytoskeletal stress fibers as mediated by the related GTPase RhoA (Jaffe and Hall, 2005). During neurulation, regulated cell motility appears essential for neural fold recognition prior to adhesion and fusion of the apposing folds. Lamellipodium-like cellular protrusions emanate from the tips of the folds as they converge in the midline (Figure 1B), a process that may require ephrin A-ephrin A receptor interactions (Abdul-Aziz et al., 2009) whose downstream effectors include Rac1. Nonneural ectodermal cells make the first contacts in the cranial and possibly also spinal neural folds (Abdul-Aziz et al., 2009), the sites of PAR2 expression. PAR1/2 may also affect the epithelial remodeling that follows neural fold adhesion (Figures 1C and 1D). Camerer et al. suggest that the action of proteases including matriptase may be required for normal remodeling, an attractive hypothesis in view of the disruption of cell-cell and cell-matrix associations that is involved and the strong association with apoptosis as a secondary consequence (Massa et al., 2009). Moreover, it might be expected that such protease activity would need to be precisely regulated, in keeping with the finding of neurulation disruption in situations of either under- or overstimulation, as demonstrated by Camerer et al. and Szabo et al., respectively.

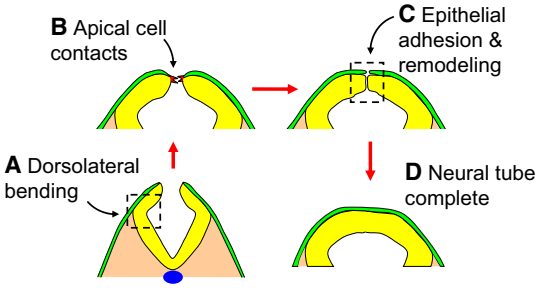

Figure 1. The Late Events of Mammalian Neural Tube Closure with Particular Reference to Mouse Spinal Neurulation

Cranial closure proceeds through a similar sequence of events, but with morphological differences: in particular the marked convexity of the elevating cranial neural folds (Copp, 2005).
(A) Bending of the neural plate (yellow) occurs at the median hinge point, overlying the notochord (blue), and at paired dorsolateral hinge points (box) where the basal surface of the neural plate changes from contact with the paraxial mesoderm (brown) to contact with the nonneural ectoderm (green). Dorsolateral bending brings the neural folds into apposition in the midline.
(B) As the neural fold tips approach each other, lamellipodial cellular protrusions (red) extend toward the contralateral fold and appear to initiate contact. Previously, these protrusions have been suggested to originate either from neuroepithelial or nonneural ectodermal cells, with possible variation along the body axis.
(C) Once fold-to-fold adhesion is established, epithelial remodeling (box) occurs in order to generate a continuous neuroepithelium and covering surface ectoderm in the dorsal midline.
(D) Neural tube closure is complete.

The potential complexity of the cellular mechanisms of morphogenetic events like neural tube closure can be mind-boggling. However, with incisive conditional transgenic analysis and step-wise definition of signaling cascades, as in these two recent papers, we can now look forward to a progressive unraveling of the secrets of the closing neural tube.

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