Mechanics of Neural Tube Morphogenesis

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Abstract

The neural tube is an important model system of morphogenesis representing the developmental module of out-of-plane epithelial deformation. As the embryonic precursor of the central nervous system, the neural tube also holds keys to many defects and diseases. Recent advances begin to reveal how genetic, cellular and environmental mechanisms work in concert to ensure correct neural tube shape. A physical model is emerging where these factors converge at the regulation of the mechanical forces and properties within and around the tissue that drive tube formation towards completion. Here we review the dynamics and mechanics of neural tube morphogenesis and discuss the underlying cellular behaviours from the viewpoint of tissue mechanics. We will also highlight some of the conceptual and technical next steps.

Keywords

Neural tube, morphogenesis, mechanical forces, mechanical properties

Introduction

The formation of an epithelial structure with an enclosed lumen such as a tube or a vesicle is a fundamental module widely employed in tissue morphogenesis throughout animal development. This deformation of the epithelial sheets enables large-scale changes of curvature and topology within the tissue [1] and is fundamental to the structure and function of epithelial organs. Furthermore, these changes carve out new surfaces and inter-tissue spaces for the development of other tissues, making subsequent structures possible and contributing to the complexity of the body plan. Notable examples of this process in early embryos include blastocyst expansion [2,3], gastrulation [4,5], neurulation [6,7], otic vesicle formation [8], and others [9]. Such deformations require the production of mechanical forces and the regulation of tissue

mechanical properties, and must operate under the physical constraints from the neighbouring tissues and the environment (such as the extracellular matrix [10–13] (ECM), the extraembryonic substrates [14,15], and the lumen [3,8]). On the cell level, it has become realized that most cell behaviours (e.g., divisions [16,17], constriction [5,18], apoptosis [19], interkinetic nuclear migration [20], junctional transitions [21], etc.) directly contribute to and respond to tissue mechanics. Our understanding of these physical mechanisms has rapidly improved recently thanks to an expanding toolkit of mechanical measurement and perturbation [22] and high-resolution/high-coverage imaging [23], raising hopes for formulating quantitative models that link genetic regulation to tissue level mechanics and morphometrics. Such models will be essential for harnessing nature's developmental mechanisms in practical applications, such as tissue and organoid engineering.

As a prominent model system of epithelial morphogenesis, the neural tube is found in all chordates and is the precursor of the central nervous system including the brain and the spinal cord. A continuous neural tube extending from the head to tail containing cerebro-spinal fluid (CSF, which fills the lumen space) is a hallmark feature of the early embryo [24]. This tube structure plays a vital developmental role in neural patterning [25], neuron differentiation [26], body axis elongation [27,28] and spinal symmetry [29]. In humans, neural tube morphogenesis takes place from the 3rd week after fertilization (5th week using the clinical gestational age) [30] and is not observable in detail *in utero* with existing technologies. Aborted human embryos at these stages are scarce and *ex utero* cultures remain challenging beyond 3 weeks post fertilization [31]. These limitations result in insufficient data on neural tube morphogenesis for human [30] as compared to other popular vertebrate models. Recent advances in mammalian organoid engineering show promise for recapitulating some aspects of body axis development including the neural tube using cultured progenitor clusters derived from stem cells, particularly when certain mechanical environmental conditions such as Matrigel of specific stiffnesses or mechanical actuation are introduced [32–35]. These advances make a closer look into human neural tube morphogenesis possible in the near future. While the processes activated in response to the *in vitro* mechanical signals remain to be elucidated, these advances clearly highlight the importance of proper tissue mechanical environment in neural tube development.

The correct formation of the neural tube is essential for human health. For example, complete failure of neural tube closure in most cases cause early embryo or fetal deaths, while partially unclosed neural tubes are more often tolerated through birth, causing neural tube defects (NTDs) which are prevalent (~1/1000 new-borns) and can lead to long-term disabilities [36,37] for the individual. Both genetic and environmental factors have been associated with NTDs in epidemiological studies [38], reflecting the interactive complexity of multiple contributing processes to neural tube morphogenesis.

To date, the most effective risk reduction method is folic acid supplementation pre- and during gestation [39–43]. Surgeries at birth or even *in utero* could mitigate the functional damage of the NTD lesion [44]. Despite these interventions, a significant fraction of NTDs remain unpreventable and/or difficult to treat [36,40,42,45]. The analysis of NTD etiology will be greatly enhanced if the mechanics of neural tube formation is better understood.

Neural tubes in different species vary broadly in size, shape and functional capacity. Correspondingly, they also show apparently distinct modes and dynamics of morphogenesis. For example, in basal chordates such as sea squirts, the larva exhibits a simple neural tube of several cells in the cross-section [46,47]. In amniotes, neural tube growth is long-sustained beyond embryonic development leading to advanced shapes such as the folds of the brain [48]. Great diversity also exists within amniotes. For example, avian embryos have larger neural plates than mammals (e.g., early neural plate widths: ~1mm in chicken vs ~400μm in mouse embryos) [49,50]. The human epiblast has a flat geometry distinct from the mouse epiblast, which is cup-like [51,52], suggesting different shapes of early neural plates within mammals. Despite these initial differences and before further structural and functional elaborations are added through later development, a closed epithelial/pseudo-epithelial tube as a dorsal component of the body axis is always achieved among the species examined, highlighting a conserved (or canalized) key developmental stage. It is the mechanics of this universal morphogenetic step that will be the focus of this review for conceptual simplicity. For the concurrent patterning of neural progenitors [53] and further growth and deformation of the brain and spinal cord [54,55], we refer readers to the cited in-depth reviews.

Approaching from a mechanics perspective, we consider the neural tube as a piece of soft matter subjected to intrinsic and extrinsic forces and mechanical constraints from neighbouring tissues and the environment. The patterned and dynamic forces act on tissue mechanical properties (which are also patterned and dynamic) to cause the deformation required for making a tube. We therefore structure the review in 3 parts that describe the components of a deformation equation: the strain, stress and mechanical properties. In the first section, we describe the modes of neural tube formation in geometry terms and the corresponding cell/tissue dynamics; in the second section, we consider the journey of a piece of neural tissue through tube morphogenesis and evaluate its experiences of different forces; in the third section, we examine the mechanical properties of the neural tissue and their regulation. We propose that a spatial-temporally resolved map encompassing these parameters will quantitatively model neural tube morphogenesis. Such a model will enable a precise comparison between normal and perturbed/mutated conditions allowing dissection of the underlying regulatory mechanisms. We will discuss the gaps between this model and our current

understanding of neural tube mechanics and highlight how recent technical innovations may be able to bridge them.

1. Morphological changes of the forming neural tube.

The morphogenesis of the neural tube has been studied since the early days of embryology [56]. During gastrulation, the neural tissue arises from the dorsal part of the ectoderm to form the tube at the stereotypic anatomic location dorsal to the notochord. Fitting the definition of a tube, the tissue either encloses or cavitates to create a fluid-filled inner lumen. These different modes of tube formation are found along the anterior-posterior axis in some single species, or when different species are compared [57]. For example, in amniotes, the anterior head and trunk neural plate folds towards the midline (appearing as a 'neural groove' [58]) and fuses dorsally to enclose a lumen [39,57] (primary neurulation). The neural plate at these axis levels is larger and generally more advanced in the progress of tube formation. In Xenopus, video recording and back-tracking show that the prospective hindbrain area spans as wide as 90 degrees from the dorsal midline, covering half of the embryo circumference [59]. Neural fates have already been specified while the tissue was a wide epithelium (neuroectoderm). In the posterior body such as the amniote tail however, a rod-like tissue ('medullary cord' [60]) composed of newly produced neural cells from the so-called bipotent neural-mesodermal progenitor (NMP) domain cavitates to make a *de novo* lumen [58,61,62] (secondary neurulation). These differences are not clear-cut and a transition zone has been described as "junctional neurulation" [63] where a mixed mode of both primary and secondary neurulation takes place along the dorsoventral axis. In the teleost neural tube, a 'neural keel' [64,65] forms whose new apical lumen surface is more dynamic than those of the amniotes and the process shows characteristics of both primary and secondary neurulation [57]. There is a possibility that these apparent differences in morphogenetic dynamics are outcomes of conserved mechanisms playing out in different tissue environments (further discussed in part 2).

The folding-based primary neurulation is responsible for brain and anterior spinal cord formation in amniotes. The extent to which primary neurulation reaches along the spinal cord differs slightly between species. In chick, primary neurulation is the predominant process until the end of the thoracic region and the start of what will become the lumbar region [63]. In human embryos the exact location of the transition is unknown, beyond closure of the posterior neuropore being located at the upper sacral level, though others have placed the shift from primary to secondary at the lumbosacral level [30,62,66,67]. In most vertebrates [57], primary neurulation begins with the initial neural plate, formed at the end of gastrulation and continuous with the surface ectoderm (SE, or non-neural ectoderm, NNE) laterally, which begins to thicken in the dorsoventral axis. Cells become taller and intercalate to aid in the shaping of the neural plate [68,69]. These cells then

begin to move towards the midline, leading to the convergent extension of the tissue [58,59]. Next, the lateral edges of the neural plate move upwards to form the neural folds, after which the midline of the newly formed groove begins to bend. Hinge points form at the midline and part way up the rising walls, which then come together at the apex to seal and form the closed neural tube [6,70]. This series of events progresses from the anterior to the posterior of the embryo, meaning the cranial neural tube forms and closes before the spinal neural tube in what may be likened to a zippering motion (Figure 1).

The caudal most aspect of the amniote neural tissue undergoes a different process called secondary neurulation to achieve the tube shape [61,66,71]. Precursor cells originate in a transient space caudal to the closing posterior neuropore termed the tailbud. Pioneering studies identified a population of bi-potentiate neuro-mesodermal cells at early stages in this region [72] and following work has again identified this group in late stages in mouse and chick [73,74]. The identity of the tailbud cells in the intervening stages, and whether NMPs are present, is still poorly understood. More recent work indicates the secondary neural tube precursors in the tailbud may be uni-fated neural stem cells until HH17 in chick [75] sitting alongside mesodermal stem cells [76] but are unexpectedly capable of producing both neural and mesodermal daughter cells from HH19 onwards [75], perhaps explaining the later population identified as NMPs. These NMPs undergo a mesenchymal-to-epithelial transition (MET) [77–79] and condense to form a solid rod, termed the medullary cord [79,80]. These cells polarize, with the central region of the cord then cavitating to form multiple small openings [7,71]. Next, these openings coalesce into a single cavity. A similar process was considered to occur in the whole neural tube of fish until recent imaging progresses highlighted some major differences. For example, while zebrafish neural tubes also show a more flexible epithelial organization with midline divisions and cell crossings [65], cell sorting [81] and a late *de novo* lumen [82] that are similar to secondary neurulation, the neural cells intercalate more globally, whereas in the chicken posterior neural tube a more gradual mesenchymal to epithelial change and spatially sequential intercalation were observed [57,83].

A recent study [7] examined secondary neurulation in avian embryos in great detail. By measuring the expression levels and distribution of T/Brachyury (marking mesodermal fate) and Sox2 (marking neural fate), it was found that the differentiation from NMPs to neuroepithelial progenitor cells happens in both a dorsoventral direction and from the periphery towards the centre of the medullary cord. The peripheral cells are surrounded by a developing basement membrane as they differentiate. In contrast, the central most cells of the medullary cord remain mesenchymal until near the end of the neurulation, with some central cells appearing to differentiate through loss of T/Brachyury whilst still retaining mesenchymal properties. Post the onset of MET small lumens of varying sizes

begin to form at a one cell distance from the basement membrane, between the newly epithelialized peripheral cells and the still mesenchymal central cells [7,84,85]. This formation of smaller lumens that later coalesce into a central large lumen [80,84,85] has also been observed in human embryos [86]. The central cells remaining between the small lumens intercalate with the epithelialized cells of the outer edges of the medullary cord, rather than disappear through apoptotic programs. This behaviour occurs immediately after cell division and requires the presence of Smad3 [7].

Given these drastic differences of morphology and cell dynamics between neurulation modes within an organism and between species, it is tempting to hypothesize fundamentally distinct underlying mechanisms. On the other hand, a set of common cellular mechanisms playing out under different tissue sizes and developmental timing is also attractive because it increases the evolvability of the neural tube. To investigate these possibilities further, we will take a deeper look into the tissue mechanics during neural tube formation. We will compare how cellular processes such as epithelialization, constriction and intercalation play out in different model systems and contexts to control the modes and dynamics of morphogenesis.

2. Driving forces of neural tube morphogenesis.

The active forces responsible for shaping the neural tube depend on cellular activities. Locally, it can be argued that any cell behaviour that involves cell deformation can lead to forces. These forces might either dissipate (in most cases) or add up and propagate, and only in the latter case can a collective tissue-level force arise and drive productive morphogenesis.

The cellular behaviours known to impact neural tube morphogenesis are wide and complex. Several major cellular processes driving neural plate shape change through tube closure have been studied extensively and their integrated mechanical action has been recently modelled [68]. First, cells in the neural plate elongate along the apical-basal axis as they become specified as neuroepithelial progenitors [69]. This causes plate-wide reduction of cellular apical surface area in the neural plate and distinguishes the tissue from the connected surface ectoderm [87]. This cellular change appears universal for neuroepithelial progenitors and does not depend on the shape or epithelial organization of the neural plate. For example, in the zebrafish neural tube and the secondary neural tube in chicken, where the epithelial organization is not well established, cells show similar behaviours individually rather than collectively [57]. Concomitantly, neural cells undergo rearrangement through polarized intercalation which thin out the tissue medial-laterally while extending it anterior-posteriorly along with body axis elongation [27] in both primary and secondary neural tubes. These are achieved by junctional remodelling and/or directed protrusion activities mediated by the

planar cell polarity (PCP) pathway [88–90]. Second, in the primary neural plate, specific cells such as the midline floor plate and the dorsal lateral areas bend to form high curvature “hinge points” causing the neural plate to fold [6,91]. For the medial hinge point (MHP), bending relies on actomyosin activity localized to the apical cortex of the cells [37,91–93]. Basal positioning of the nuclei and apoptosis also contribute to reduction of the apical surface [19,91,94,95]. These processes together generate the intrinsic force that drives the bending of the neural plate towards a tube and are best demonstrated in its ability of driving the folding of isolated neural plate explants in different model systems. However, the apical tension increase does not appear to drive enough folding to enable full closure in neural tube explants from avian embryos [96,97], which could be due to loss of other contributing forces such as recently identified compression from the flanking presomitic mesoderm [28]. The role of the connected SE remains controversial [98,99]. Its convergent movement may provide a pushing force to assist neural folding [98] while its tension could be a resistance [14,100]. Under the joint effect of these forces, the neural folds on both sides eventually move into sufficiently close proximity for the “zippering” process mediated by cell protrusions and junctional dynamics to kick in [47,101]. This conserved mechanism [102] may also promote the movement of unclosed parts towards the midline by generating a pulling force from the open neuroepithelium just beyond the zippering point [103]. A study of the basal chordate *Ciona intestinalis* found that rapid junctional contraction immediately posterior to the advancing zipper is caused by myosin II and draws the neural folds together to allow for the progression of the zipper, whilst cell contact rearrangement immediately anterior to the zipper reduced tissue resistance [47]. The secondary neural tube does not show distinct hinge point formation on the tissue level however the coalescence of the *de novo* lumen means that the apical surface of the polarizing cells must reduce [57]. Whether a more disorganized apical constriction and/or intercalation behaviour drives lumen formation and consolidation in the secondary neural tube remains to be tested.

To contextualize these behaviours and illustrate the forces they produce, we will first focus on a unit piece of the neural plate tissue (Figure 2). This unit tissue moves in space from its neural specification in the ectoderm or the NMP domain to its final location in the tube structure, driven by a net body force. In both the lab reference frame and a fixed anterior landmark (such as a somite) in the embryo, the tissue will exhibit a net movement consisting of an anterior to posterior, a lateral to medial, and a ventral to dorsal component, respectively (note that as the neural tube forms, the apical-basal axis of the neuroepithelial tissue will rotate in relation to the dorsal-ventral axis of the embryo, to form an angle with the dorsal-ventral and medial-lateral axes, depending on its location on the tube). In the meantime, the unit tissue also undergoes deformation, which includes thinning, elongation and curving, driven by a combination of internal and external stresses. Depending on the location and developmental time of the tissue, its

mechanical properties and stress experience will vary, reflecting mechanical patterns in the tissue. Given the significant differences in tissue architecture between primary and secondary neurulation, we will discuss them separately but will compare and identify common cellular mechanisms of force production (Table 1).

2.1 Forces from cell movement and rearrangement: Convergent extension by cell crawling and junctional contraction

The unit tissue shows deformation throughout its journey in neural tube formation. The most prominent component of the deformation is convergent extension driven by internal stresses generated from polarized cell intercalation, which causes the tissue to narrow along the apical-basal axis whilst extending antero-posteriorly [59]. In neural plate tissue, the intercalation movements rely on the PCP pathway which provides spatial polarity [64,89,104–106]. In a Xenopus study, the PCP components and the cytoskeleton machinery responsible for intercalation are found to be strongly associated [107]. They may be linked by the Rho / Rho associated protein kinase (ROCK) pathways however the exact linking mechanism remains unclear [104]. Generally, ROCK kinases influence cytoskeletal tension through the formation of stress fibres and focal adhesions via the phosphorylation of myosin II and drive cell shape changes in many different contexts [108]. Two apparently distinct mechanisms of cell intercalation have been described: cell crawling and junctional contraction. Cell crawling behaviour is typified by the intercalation of mesenchymal cells using polarised actin-based protrusions, which emerge from the mediolateral vertices of the cells. These protrusions form a moving front of lamellipodia on the leading edge of the mobile cell mass [109]. Contractile actomyosin behind the leading edge connects to the ECM via focal adhesions, resulting in a traction force that moves the cell forward [110,111]. Junctional contraction, in contrast, causes cell intercalation through the shortening of cellular junctions between anteroposterior neighbouring cells by polarised junction remodelling [105,107,112,113]. This involves actomyosin contraction, which happens in a ratchet-like mechanism where RhoA oscillatory signalling allows for the compression and stretching behaviour to overcome the compression limit of 80% on the contractile middle third of the junctions [114]. Interestingly, these oscillations in contracting junctions appear to correlate to relative enrichment levels of PCP proteins such as Prickle2 and Vangl2 [107,115] which are known to underlie neural tube morphogenetic defects.

These two behaviours were previously thought to be completely distinct, as the cell types in which they were first described differ significantly in both cell adhesion properties and polarity. However, recent discoveries show the two processes appear to occur in conjunction in multiple cell types, including during Drosophila germ band extension [116] and in the mouse neural plate [117]. Time lapse analysis indicates that neural plate cells form cell protrusions on their basal surface while undergoing junctional

remodelling on the apical surface. The basal protrusions form most often at tricellular junctions but also on lateral edges between cells. Individually these protrusions are multipolar but looking at the tissue as a whole there is a clear mediolateral bias [117]. The basal surface of the cells elongates, and cells change their aspect ratio to a highly mediolaterally biased shape. This elongation of the cells, despite being perpendicular to the axis of extension and thus not directly contributing to the process, is still required. This bias is potentially due to the asymmetric localisation of myosin IIB at the anterior and/or posterior ends of the cells. The apical surface of the cells undergoes remodelling and neighbour exchange via different methods of boundary rearrangement. These include T1 formations [112], single cell intercalation, division and rosette resolution [118]. When cells exhibiting rosette resolution are tracked to see whether they resolve the basal or apical rosette first, it was found that the number was roughly equal, indicating that both protrusive and junctional mechanisms contribute significantly to the overall intercalating behaviour, although the isotropic distribution of myosin IIB apically suggests that the direction of intercalation is driven mainly by the protrusive basal aspects [117]. This finding in the mouse model was corroborated by a recent study in Xenopus [119] where the authors used the oscillatory nature of the junctional contraction to assess the relative roles of crawling and contraction in cell intercalation. They further show that cell adhesion is necessary for the integration of these two disparate mechanisms, and that cell movement is more efficient when both are working in concert.

The polarized intercalation driven by these cellular mechanisms occurs in almost the whole tissue throughout neurulation, being observed in the resolution of the central lumen of secondary neurulation [7] as well as its role in primary neurulation as described here. This internal force is therefore the most consequential in controlling the neural tissue shape. Consistently, mutations of the PCP pathway in fish and mammals cause wide neural tubes with multiple apical surfaces and NTD-like phenotypes [64,65,101,117], and the related genes have been associated with human NTD cases [38,120]. However, the thinning and elongation of the unit tissue under the intercalation stress do not yet produce curvature needed in a tube shape. Another major force is needed to create the neural folds.

2.2 Forces from cell shape changes: Apical constriction and interkinetic nuclear migration

Following convergent extension, neural folds form as the lateral walls of the neural tube begin to rise. This bending of the plate to allow elevation of the sides is aided by the formation of a MHP, followed by two dorsolateral hinge points (DLHP) [58,121]. The cells in the hinge regions acquire a wedge shape through reduction of apical surface area, caused by apical constriction and a delay in interkinetic nuclear migration

[87,93,95,122]. These mechanisms are better established for MHPs but not clear for DLHPs and other mechanisms could play a role. Unlike cell intercalation, the reduction of apical surface is not tissue-wide but spatially specific in the hinge point regions. In these regions, actin and myosin can be observed to accumulate along cell junctions and also the medial cortex near the apical surface. As a general mechanism, the myosin drives a ratchet-like pulsatile contraction [5,6] to reduce the apical surface area, causing a tissue-level increase of apical tension that subsequently drives epithelial bending. The tension is progressively relaxed following tissue shape change and is not required to maintain tissue shape.

At the start of primary neural fold bending in mice, the cells at the midline of the neural plate experience a prolonged S phase as nuclei remain basally located, induced by signals from the notochord [6,93,123]. This pause before G2 also puts the process of interkinetic nuclear migration (INM) on hold, as the ascension of the nuclei to the apical surface of the cell is dependent on the active movement of microtubules during G2, triggered by the microtubule associated protein Tpx2 [20]. INM is associated with changes in apical cell surface area, with the apical surface narrowing during G1 as the nucleus moves basally [20]. This is followed by a period of surface stability during the S phase where the nucleus remains basally located. During G2 the nucleus ascends to the apical surface and increases the apical surface area again [124]. However, with the nuclei remaining basally located in the midline cells, the basal surface area remains roughly the same whilst the apical surface decreases in size. Coupled with the constriction caused by the pulling of actomyosin cables between apical tight junctions, this results in a drastic shrinking of the apical surface area that gives the cells a wedge-like shape. This shape change enables formation of the hinge points [6]. It also occurs more broadly across the neuroepithelium to reduce its apical dimension and promote progression towards neural tube closure [93].

In the secondary neural tube, no discernible hinge points form. Yet apical constriction and interkinetic nuclear migration may be playing a similar mechanical role in a less organized manner to drive cavitation and tube formation. These shape changes are carried out by individual neuroepithelial progenitors that derive from a proliferative pool of NMPs at the posterior end of the embryo in amniotes [74,125–127]. The NMP pool is found in the tailbud, which is composed of irregularly shaped cells arranged in a dense network that intermingles with cells of neighbouring regions [85]. A hallmark feature of these bi-potential NMP cells is their co-expression of T/Brachyury and Sox2, with the neuroepithelial progenitors they differentiate into in the medullary cord exhibiting a downregulation of the T/Brachyury whilst retaining Sox2 expression [128–130]. This differentiation drives a mesenchymal to epithelial transition (MET) that change the cell shapes from irregular polygons to elongated, in line with a basal shift of the nucleus and a rearrangement of the apical membrane consistent with interkinetic nuclear migration

and apical constriction [7,131,132]. Recent progresses in the generation of NMPs *in vitro* now provide a better understanding of the gene regulatory networks that control the state changes of these cells as they differentiate towards the posterior neural tube [133–135]. A tailbud organoid will be of great value for further dissection of the cellular mechanics responsible for this part of neural tube formation.

The creation of an inner apical surface, either in the primary or the secondary neural tube, means that the tissue must form a curved surface. The forces discussed here, generated by the cytoskeleton machinery in a polarized manner appear to be the predominant (if not only) mechanism for the unit tissue to bend. It is worth noting that these forces also drive tissue movement. As tension transmits along the apical surface, a torque is generated on neighbouring tissues, causing them to rotate dorsal-medially towards making the tube enclosure.

2.3 Forces from neighbouring tissues and the lumen

It is believed that the tension from the hinge points accounts for most of the folding force in the primary neural tube. Supporting this notion, in chicken neural tube explants, the MHP still forms and drives folding. However, the DLHPs are affected without the SE and the tube formation is incomplete [96,97]. These suggest a role of neighbouring tissues in providing signals and/or forces to assist folding. In all examined model systems, the neural plate and the SE remain connected until the very late stages of neural tube morphogenesis. As the neural plate converges towards the midline, the SE follows closely. The cell states and mechanics near this connection are found to be important for tube closure in some regions of the mouse neural tube. For example, expression of Ghrl2/3 in the SE needs to be tightly controlled to prevent changes in adhesion molecules and actomyosin activities that can disrupt tube closure [136]. Studies in chick and amphibians also show that the SE plays a constructive role to ensure the complete closure of the neural tube [98,137]. In Xenopus, integrin β1-dependent dorsal-medial migration of SE deep layer cells is proposed to assist the closure [137]. In chicken embryos, removal of claudins specifically expressed in the SE, which are important for tight junction function and epithelial integrity, causes failure of folding and NTDs [138]. It was proposed that the convergent movement of the SE could push the neural plate to help DLHP formation and folding [97]. On the other hand, tension between the neural plate and SE and other tissues may resist the folding and convergent movement. In a recent study in chick [14], when the tension is artificially maintained at a higher level on the vitelline membrane and extraembryonic tissues, neural folding delay and some cases of failed closure are observed. Similarly, in mouse mutant embryos where endoderm and notochord (but not the neuroepithelium) over-proliferate causing body axis curvature change, neural tube closure is affected likely through its mechanical

connections with these tissues [87,139–141]. These inter-tissue mechanical interactions remain to be further characterized with *in vivo* measurements.

The role of the underlying paraxial mesoderm which flanks the neural folds was also unclear. In the mouse Tbx6 mutant, where the presumptive mesodermal cells adopt a neural fate instead, the paraxial mesoderm becomes additional neural tubes [142], indicating tube formation does not require the mesodermal cell state *per se*. Nonetheless, as a flanking tissue that is also undergoing shape changes, the possibility remains that the paraxial mesoderm has an impact on the neural plate/tube. Indeed, it was found that during zebrafish neurulation, the mesoderm couples with the neural plate through ECM components fibronectin and laminin [143]. This ECM connection ensures coordinated movement and shape changes of both neural and mesodermal tissues. In chick, it was found that the posterior paraxial mesoderm undergoes tissue level expansion associated with high cell motility driven by fibroblast growth factor (FGF) signalling [144]. This behaviour was found to be essential for body axis elongation and was proposed to be a force generation mechanism. While how cell motility causes tissue expansion is still unclear, the close proximity of the tissue to the neural plate and the SE suggests a force normal to the epithelial layer might be felt by the tissues. In a recent study [28], soft alginate gels were implanted to replace the posterior neural tube and a compression from the paraxial mesoderm was detected. Without the mesoderm, the convergence of the neural folds becomes delayed and could leave an open section of the tube even after the mesoderm has recovered from tissue surgery. The compression is not detected at the level of the anterior paraxial mesoderm or the somites, where interstitial space is observed between the neural tube. These results show that the paraxial mesoderm assists the convergence of the neural folds in the posterior body axis through compression. Whether this force also helps the dorsal rise of the neural fold-SE continuum and the formation of the DLHPs remain unclear.

Another poorly understood tissue-extrinsic mechanical player in tube formation is the lumen, which is known to maintain a hydrostatic pressure once the tube closes that contributes to later growth of the tube [145]. In the context of secondary neurulation, the mechanism of lumen emergence likely involves cell mechanics and pressure regulation. It was found that failed intercalation in the secondary neural tube resulted in the inability of lumen coalescence and subsequent blockage of fluid flow in the lumen [7], leading to pressure mis-regulation and tissue deformation. Mis-regulated lumen pressure has been suggested to underlie some subtypes of NTDs [146] by causing rupture and reopening of already closed neural tubes but a model testing this idea is not yet established. The dynamics of the lumen pressure are yet to be closely measured and modulated for direct tests of these possibilities.

These recent results suggest an integration of multiple forces driving the convergence and folding of the neural tube. The unit tissue experiences a combined body force from multiple pushes and pulls from different directions (Figure 2). How these forces impact each other through mechanosensing and cellular responses remain unclear. In normal conditions, their coordinated dynamics ensure the folds meet along the dorsal midline. Here, the neural tissue needs to break from the SE and fuse to create an isolated lumen surface, altering the topology of the tissues. This cannot be achieved with any of the forces described earlier and would require yet another specific force created by a distinct cellular mechanism.

2.4 Forces from cell protrusions and contacts: Fusion and closure

At the dorsal apex, the neural ectoderm is still continuous with the SE, forming a two layered structure. In order for the neural tube to fully seal and be protected from the external environment, these connections must sever and the four new tissue edges recombine in a different configuration: neural folds to join into the completed tube and SE to join as a comprehensive epithelium. The cells on the opposing SEs have been observed to produce cellular protrusions prior to fusion in several cases; membrane ruffles have been documented at the edge of the neural folds in amphibians [147], birds [148] and in mammals [149]. Filopodia have also been observed in mice [150].

In Xenopus neural folds, which are 2-layered up to the time of closure, the cells in the superficial layer apically contract creating a local rolling movement which is proposed to pull the SE medially [109]. Deep layer neural cells then employ protrusions to intercalate with the superficial layer, forming the single layered pseudostratified neural tube. These cell behaviours are crucial for neural tube closure in Xenopus and represent an intersection between the PCP pathway and cell mechanics, which is extensively covered in a recent review [104]. In amniotes, the epithelial fusion of the neural tube and the overlying SE occurs sequentially between closure points, rather than near simultaneously as in amphibians [37]. These closure points are biomechanically distinct sites along the closing neural tube where the two opposing sides meet earlier than the rest [37]. The number of closure points as well as their locations differ somewhat between species [151–153], but in most cases the fusion of these closure points leave open tracts between them referred to as neuropores which then close through a zippering process from either or both closure points at either end [37].

Mechanisms observed in other examples of epithelial fusion and previously proposed in the neural tube include purse string models and cell crawling behaviours [12,103,154]; the former involves the contraction of an actomyosin cable across the leading edge cells of the open neuropore [12] and the latter cell movement wherein both apposed cell sheets adhere to the same basement membrane and use lamellipodial protrusions to

pull themselves towards each other. The question mark over this latter proposal is the initial lack of basement membrane in the gap at the apex of the forming lumen; the epithelial leading cells do not have a basement membrane to anchor to and pull across but rather extend processes across a cavity. Further, whilst there is evidence in mice to show the existence of surrounding supracellular actomyosin cables capable of contracting as is seen in the purse string model, the timing of their appearance differs between neuropores. In the posterior neuropore they are only observed fully encircling the neuropore at later stages of neurulation, whereas in the murine hindbrain neuropore they are present from formation of the neuropore [103,154]. Thus, there is likely a combination of these processes and possibly further, as yet unidentified, components in this morphogenetic system.

In a recent study [12], local integrin mediated focal anchorage to a common basement membrane is found to be a key part of this zippering mechanism. This novel basement membrane forms at the interface between the SE and the dorsal neuroectoderm (the acute angle of the DLHP) as the dorsal neuroectoderm changes basal contacts from the paraxial mesoderm to the SE [6,12]. At the main fusion points, the cells undergo proximal junctional shortening. This is caused by local integrin β1 activation, allowing for focal adhesion to the newly deposited basement membrane. The focal adhesion acts as a centre point, above which a temporary rosette forms as the junctions between cells shorten. This pulls neighbouring cells closer together, putting apposing cells in contact with each other and enabling neighbour exchange and sealing. Whether the pulling forces generated by junctional shortening propagate further to impact neural fold movement remains unknown. Understanding the combined effect of these forces will be important to explain the unit tissue's trajectory towards posterior, dorsal and the midline (Figure 2) at the same time.

2.5 Other forces: Cell growth, proliferation, death and differentiation

Significant tissue growth accompanies neurulation. These are not only fuelled by newly specified neuroepithelial cells from the posterior progenitor domain such as the NMPs but also cell proliferation within the neural tube tissue [155]. As a contributor to tissue size, cell divisions can be expected to affect tissue shape as well. In the curly-tail mouse mutant, inhibiting cell divisions can have differential effects on neural tube closure that depends on timing and location [156]. In a recent study [157], the folate pathway is found to increase progenitor proliferation to compensate for cell loss due to neuron differentiation in a mouse NTD model. Cell death was also proposed to play a role in hinge point formation and epithelial fusion. For example, to break the continuity between the neural fold and the SE in mice, apoptosis triggered by anoikis (cell death due to a lack of anchor to an underlying basement membrane or matrix) may be needed [158]. In chick embryos, inhibiting apoptosis can cause failure of neural tube closure [159]

presumably due to a defect in the formation of the DLHP. However, in another mouse study, while apoptosis is also found to be present during the bending and fusion of the neural folds, it appears dispensable for neural tube closure as evidenced by normal neurulation in inhibitor treated cultures [160]. Mutants with downregulated apoptotic pathways such as *Casp3* [161] and *Apaf1* [162] therefore likely acquire NTD phenotypes (at the mid- and hindbrain level) through other changes rather than cell death in the neural tissue [160]. Excessive apoptosis in mouse mutants such as *mdm4* [163], on the other hand, likely causes NTDs by reducing tissue mass below the level required for tube morphogenesis.

For the unit tissue, the intuitive mechanical effect of cell growth and proliferation will be to cause tissue expansion. Cell death and extrusion, on the other hand, would be expected to shrink the tissue, creating a local contracting force (Figure 2). The mechanical consequences of these processes are less intuitive. Cell division, for example, can act to reorient neighbouring cells and influence their fates in the zebrafish neural tube [164]; Under high tension, cell divisions can also reorient to function as a relaxation mechanism [17]; losing a cell from the epithelial surface by cell death or live cell extrusion can cause a local apical tension increase and act as a response to crowding [165–167]. These observations indicate that epithelial tissues use cell number control as a responsive mechanism to mechanical forces. In the complex environment of the forming neural tube with all the different forces we discussed above interacting on the unit tissue, such a control mechanism might play a vital role to ensure morphogenetic robustness.

3. Tissue mechanical properties of the neural tube.

For forces to productively drive tissue shape change, it is essential that the tissues allow deformation during the morphogenetic stages. In addition, once a desired shape is reached, it will be beneficial for the tissues to dissipate the forces or stiffen so as to consolidate the morphogenetic progress. This change of mechanical properties is analogous to the temperature-controlled shaping of glass and metals. In the developing vertebrate body axis including the neural tube, a probably coordinated or closely-related posterior to anterior increase of epithelialization [155], ECM density and organization [10,144] and tissue stiffness [21,168,169] has been observed in multiple model systems, which is consistent with the idea of tissues holding formed shapes after the major shape changes are complete. The mechanisms that regulate tissue mechanical properties are therefore equally important for morphogenesis as the actively generated forces. Some known mechanisms already show great complexity, and likely many more are unknown. Here we will skim the surface of the field of tissue mechanical properties and limit our discussion on a few classic and recent examples in relation to neural tube formation to provide a contextualized overview. Much remains to be understood to

create a systematic picture of mechanical property regulation during tube morphogenesis.

3.1 Structural basis of tissue mechanical properties in the neural tube

Early embryonic tissues acquire their mechanical properties from multiple sources dominating at different spatial temporal scales [170]. For morphogenesis, the most relevant contributors are the cell cortex regulators, cell-cell junctions, the ECM and cell arrangements since changes at these levels underlie the viscous behaviour that underlies the mesoscopic (orders around 100μm) tissue deformation over minutes to hours. On shorter time scales, the tissues show elastic behaviour and are generally robust to mechanical fluctuations. This is because the structural components maintaining the tissue shape need time for biochemical changes to modify them.

The actin cytoskeleton is a network of polymerized F-actin stress fibres well known to resist external forces to maintain cell shape [171], hold cell integrity through isometric tension [172] and also drive cellular shape change and movement through remodelling. From a materials perspective, the organization of this F-actin structure lends itself to being a gel capable of varying states between liquid and solid depending on factors such as the type and degree of cross-linking; this affects its properties and how forces are conducted through it [173]. These forces are transmitted between the actin cytoskeleton (and through it the cells nuclear machinery) and the underlying ECM by mechanosensitive integrin based focal adhesions. These focal adhesions are formed of a wide array of proteins including vinculin and alpha actinin, the latter of which increases sharply in expression levels over the course of Xenopus neurulation [174,175] and is thought to also play a role in crosslinking and stabilization of the F-actin network [175,176]. These changes may be important in stabilizing the neural tube shape against opposing forces such as tension from the SE.

The ECM in the neural tube is also heterogeneous and dynamic. For example, the isoform of laminins present differs across the neural tube and rapidly switches expressions through development in mice [177]. More specifically the ratio of isoforms deposited by the mesoderm into the basement membrane surrounding the neural tube changes significantly from rostral to caudal [178]. Exencephaly (a form of NTD from failed cranial neural closure) is observed in 60% of mouse embryos lacking laminin a5 suggesting its expression may be key in ensuring tissue integrity [179]. At the closing dorsal apex where the neural folds meet, ECM in the basement membrane around the neural tube and the SE undergoes reorganization and remodelling [180] probably to fluidize the tissues for the fusion to occur. The ECM is a viscoelastic structure, with the elasticity given in part by fibrous proteins such as elastin and collagen and the viscosity conferred through proteoglycans, which allow for interfibrillar slippage when under

tensile load [176]. These proteoglycans include a sub-family of heparan sulphates, some of which have been implicated in integrin modulation and cell proliferation in the neural tube, such as syndecan 4 in zebrafish [181] and glypican 1 in mice, the inhibition of which leads to decreased brain size through decreased FGF signalling [182].

Cell states and arrangements also alter tissue mechanical properties. The mesenchymal to epithelial transition (MET) in the secondary neural tube likely stiffens the tissue to maintain the rod/tube shape. An improperly polarized neural plate (such as those with perturbed PCP pathways) may become thicker [64] thereby showing an increased bending stiffness that resists folding. The adhesion molecules neural cells express could also alter tissue connectedness and interfacial tension within the neural tube or between the SE. Classic observations [183] suggest the switch from E-cadherin to N-cadherin during neurulation enables the separation of the neural tube and the SE. Inside the neural tube, a recent study in zebrafish [184] showed that differential combination of adhesion molecules mediate cell arrangements between different neural progenitor domains. This mechanism stabilizes the tissue pattern and possibly shape as the cell mixing resulting from the large-scale tissue movement slows down towards the completion of neurulation.

How different layers of the mechanical structure interact with each other remains poorly understood. Cells perceive environmental mechanics and produce responses through mechanotransduction [185]. The inputs come through the ECM which is connected to various cell structures [186]. For example, all of the forces described earlier require some kind of cellular or extracellular transmission machinery, be it an actin cytoskeleton to constrict a surface, focal adhesions to anchor to the basement membrane to exert traction force or microtubule-based movement of a cell nucleus. These in many cases work in concert [187]. Neural cells respond to mechanical and chemical signals from the ECM in a variety of ways [188]. How these responses in turn modulate the mechanical properties of the neural tube remains to be investigated.

3.2 Measurements of mechanical properties of neural tissues

The actual direct measurements of neural tube mechanical properties have mostly been made in amphibian systems thanks to the large tissue sizes and external development. In an axolotl study [189], an elegant wire setup was used to measure the Young's modulus of the neural plate and SE of the embryo. The wires are pre-calibrated so that their deflections can be related to forces. By gluing them to the tissue and pulling on the explants, the stress-strain curve can be recorded for calculating tissue stiffness (~20 Pa). In Xenopus gastrulating embryos, dorsal isolates (explant of the dorsal surface including the 3 germ layers) were subjected to compressive tests where tissue deformation is detected through a calibrated optical fibre. These measurements show a

gradual stiffening of the axial tissues [168,190]. The neural plate modulus was estimated to be around 40-60 Pa.

In a more recent study [174], the explant is found to stiffen 150% in the 5 hours it takes from early neural plate to post neural tube formation stages. Interestingly, this increase in Young's modulus is not dependent on or directly caused by the changes in tissue architecture; scrambled explant that mixed the three germ layers and thus did not form the standard architecture (no convergent extension or neural fold formation) show similar modulus values. However, the scrambled tissues do not reach the correct stiffness after the same amount of developmental time. These results suggest a combination of a baseline stiffness and a capacity to stiffen via tissue organization underlie the mechanical dynamics of the tissue. The baseline stiffness is likely dependent on the cytoskeleton and rho-kinase mediated regulation of myosin activity. Consistent with this, ROCK inhibition reduces the stiffness of the overall tissue by a margin of 39-52% [174].

While providing valuable insights and demonstrating the dynamic and regulatory nature of mechanical properties of the neural plate/tube, measurements based on tissue explants have some limitations. The surgical surfaces likely have different surface tensions than the endogenous condition and may affect the bulk properties of the tissue [191] (e.g. making the neural plate stiffer due to tension increase at the cutting surface). The spatial heterogeneity is not yet well-resolved. In addition, tissue mechanical properties are dependent on time scales. A frequency based rheological approach [192] will be helpful in expanding the measurement spectrum.

Conclusion and perspectives

In summary, tissue forces and mechanical properties have a constant presence throughout neural tube formation and must be regulated properly spatially and temporally by different cellular and molecular mechanisms. Disruptions of any of these processes during any of these stages could result in a final outcome of an unclosed portion of the neural tube. Such disruptions act as effectors of genetic dispositions and environmental factors that cause NTDs in humans, making it important to understand tissue mechanics in the effort of preventing and alleviating NTD conditions. Recent advances have brought us significant new insights on multiple fronts of neural tube mechanics. Strikingly, we still do not know the magnitudes and dynamics of the different types of stresses or mechanical properties of the forming neural tube or its neighbouring tissues for most model systems, and little high-resolution, high-precision measurements have been done *in vivo*. We also have limited knowledge of the lumen pressure and its regulation, or a detailed understanding of the ECM structures and organization. Cellular dynamics, particularly for the dense part of the tissue such as the secondary neural tube

and model systems that are more difficult to access, remain undocumented by live imaging. This lack of quantitative information in both mechanics and dynamics acts as a major roadblock towards the construction of rigorous physical models [193] that can account for the complexity of the tissue geometries and the multitude of forces during neural tube formation.

Emerging technology provides optimism for overcoming these challenges. Classic mechanical measurements of tissue force and mechanical properties such as cutting [58], glass fibres [194], and embedding gels [28,168] have been informative. More recently, methods that can be applied in intact, live embryos are being developed to overcome some of the limitations of more invasive methods [22]. These new methods include magnetically controlled droplets [170], atomic force microscopy [195] (AFM), Brillouin microscopy and optical coherence tomography [196–198] and optically trapped nanoparticles [199]. The droplets provide a local and dynamic measurement of tissue mechanics leading to the finding of solid-fluid jamming transition in the paraxial mesoderm of zebrafish [21]. AFM provides excellent spatial resolution but is limited to tissue surface. This could be complemented by 3D approaches such as elastography with improved resolution [200]. While mechanical properties have become better understood with these methods, *in situ* measurements of the bulk and surface stresses discussed in this review remain challenging for the neural tube. It is also important to extend the capacity of these methods beyond measurement to enable precise mechanical perturbations. Alternatively, mechanical probes can work in conjunction with and help calibrate genetic methods of mechanical perturbation, such as optogenetically controlled cell mechanical regulators [201]. Imaging continues to play a key role in understanding neural tube morphogenesis. Recent studies have performed precise measurements of tissue shapes and volumes [155,202] providing the required quantitative information to evaluate different mechanical hypotheses. In model systems like zebrafish, light-sheet imaging provides unprecedented coverage of single-cell dynamics throughout neural tube formation [203]. In the less accessible amniote models, improved culture methods and imaging set-up are also quickly improving the cellular coverage [12,49] but more remain to be done such as for the secondary neural tube. The quantification of these usually very large datasets now requires imaging analysis algorithms based on deep learning [204]. High quality imaging data provides an alternative and complementary approach to contact-based methods of mechanical measurements through mechanical inference with physical models [205]. Together, these interdisciplinary innovations promise to unravel new layers of the mechanisms of morphogenesis and improve our capacity of controlling tissue shape in engineering and medical settings not only for the neural tube model but also epithelial development in general.

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Figure Captions and Tables

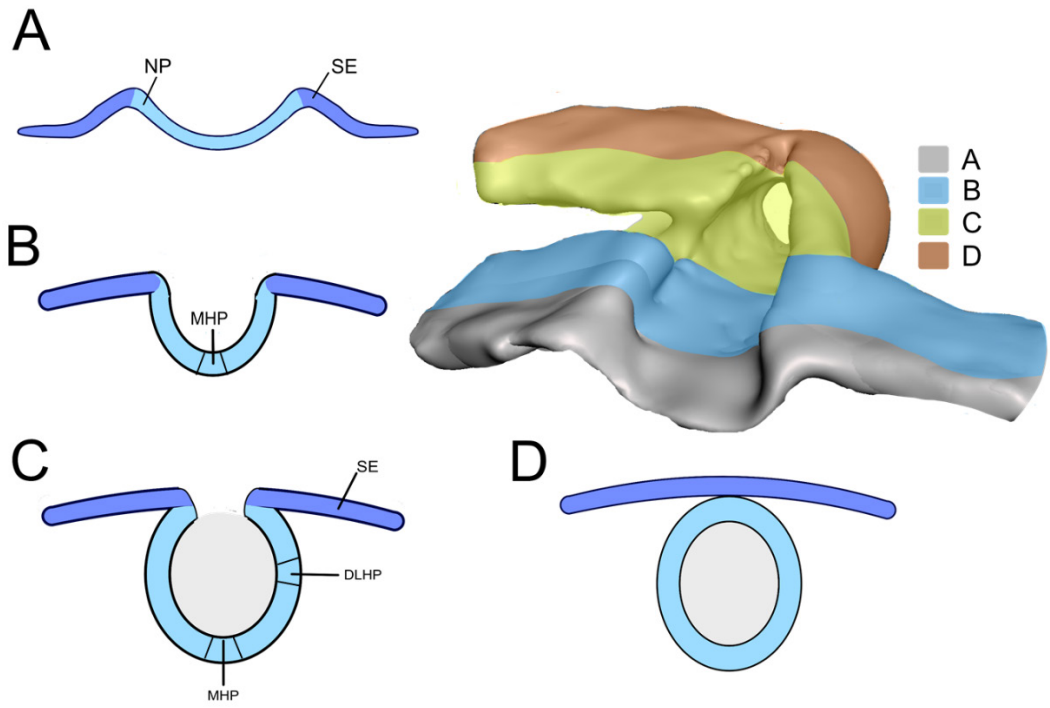
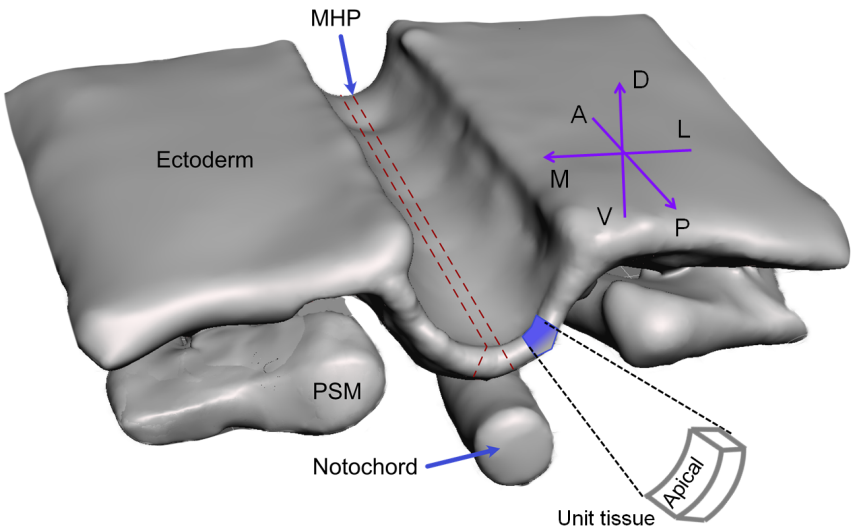


Figure 1. Cross-section and 3D illustration of primary neurulation. Panels A to D represent the major stages of tissue deformation across primary neurulation. The 3D rendering shows the posterior (near) to anterior (far) progression of neural tube formation to illustrate the continuous changes along the antero-posterior axis and how these different stages transition into one another. A cut-out has been made between B and C in the 3D render to illustrate the bending structure of the DLHP (dorsolateral hinge point) whilst highlighting the tissue as a continuum with the SE (surface ectoderm). NP (neural plate); MHP (medial hinge point).



Process	Force	Type	Illustration	Effect	Combined Effect
Anterior neural tube elongation	A to P push	Ext.		A-P movement	The unit tissue moves posteriorly, dorsally and medially throughout neural tube formation
Hinge points formation	pull along the neuroepithelium	Ext.		V-D, L-M movement	
SE movement	M to L pull	Ext.		M-L movement	
PSM expansion	push on the neuroepithelium	Ext.		V-D, L-M movement	
Apical constriction	tension on the apical surface	Int.		bending	The unit tissue bends towards the apical side, increases curvature towards a tube shape
Interkinetic nuclear migration	push on the apical surface	Int.		expansion, flattening	
Cell extrusion/death	pull towards the extrusion point	Int.		bending	
Apical-basal elongation	pull along the neuroepithelium	Int.		thickening	The unit tissue expands, primarily by extending along the AP axis
Radial intercalation	push along the neuroepithelium	Int.		thinning, expansion	
Medial-lateral intercalation	push along the AP axis	Int.		narrowing, elongation	
Growth and proliferation	push along the neuroepithelium	Int.		expansion, elongation	

Figure 2. Forces driving neural tube morphogenesis. Forces that drive the movement and deformation (bottom table) of a unit tissue from the neural plate in the multi-tissue environment (top) are visualized. The unit tissue is the subject of mechanical analysis and undergoes both movement and deformation, under the

combined effect of external (Ext.) and internal (Int.) forces. The table include speculations and may not cover all relevant processes and forces. PSM (presomitic mesoderm or paraxial mesoderm), MHP (medial hinge points), ECM (extracellular matrix), SE (surface ectoderm), A, P, D, V, L, M refer to anterior, posterior, dorsal, ventral, lateral and medial, respectively.

Table 1. Comparison of tissue and cellular processes in primary and secondary neurulation.

Cell/Tissue Processes	Primary Neurulation	Secondary Neurulation
Actomyosin cable: purse string model	Contraction of an actomyosin cable around the leading edges of the open neuropore to close it [12], observed in mouse hindbrain and latter stages posterior neuropore [37,154].	Unlikely to be present due to lack of neural folds
SE convergence and mechanical integrity, PSM convergence	Important for proper closure. SE required for DLHPs in chicken anterior neural tube explants [96,97]. SE specific expressions of Ghrl2/3 [101,136] and claudins [138] mediate closure in chick and mice. PSM is proposed to assist neural fold convergence via compression [28] in chick.	Unknown. The connection between SE and the medullary cord is not well characterized. Whether the mesodermal progenitors from the NMP domain affect neurulation is unclear.
Apical constriction	Occurs during bending of the plate; a reduction in apical surface area over the basal surface area results in a wedge-shaped cell, causing curvature of the plate [87,93].	MET includes cell polarization and elongation consistent with apical constriction. Evidence of active constriction needed.
Apicobasal cell elongation	Occurs in the early neural plate, as cells become specified as neuroepithelial progenitors [69,70]. Collective.	Occurs during cavitation when cells start to show neuroepithelial characteristics [61]. Individualized.
Cell Intercalation	Occurs broadly throughout the neural plate and drives tissue shape change. This rearrangement thins out the tissue medial-laterally and extends it antero-posteriorly in line with body axis extension [27,88]. Regulated by PCP.	After the formation of multiple smaller lumens inside the medullary cord, the central cells between the lumens intercalate with the outer epithelialized edges of the cord to allow resolution into a single large lumen [7]. Regulated by PCP.
Interkinetic nuclear migration (INM)	The apicobasal movement of the nucleus results in narrowing of the apical surface in G1, contributing to the bending of the plate [20,93]. This is linked to cell cycle delays at the hinge points, where a pause in S phase induced by the notochord sees the nuclei remain basally located [95].	Whilst not necessarily the classical representation of INM, changes in cell shape that occur during MET occur in line with a basal shift of the nucleus and a rearrangement of the apical membrane consistent with INM and apical constriction [7,133,134].
Mesenchymal to epithelial transition (MET)	Unlikely to be present due to the primary neural plate arising directly from the epiblast that is already epithelial.	Differentiation of the NMP progenitors in the medullary cord causes a shift from irregularly shaped unpolarised cells to polarised epithelial cells [7,131]. This occurs from the periphery of

		the medullary cord and moves inwards towards the centre [7].

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