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Mechanisms of vertebrate neural plate internalization

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ABSTRACT The internalization of multi-cellular tissues is a key morphogenetic process during animal development and organ formation. A good example of this is the initial stages of vertebrate central nervous system formation whereby a transient embryonic structure called the neural plate is able to undergo collective cell rearrangements within the dorsal midline. Despite the fact that defects in neural plate midline internalization may result in a series of severe clinical conditions, such as spina bifida and anencephaly, the biochemical and biomechanical details of this process remain only partially characterized. Here we review the main cellular and molecular mechanisms underlying midline cell and tissue internalization during vertebrate neural tube formation. We discuss the contribution of collective cell mechanisms including convergence and extension, as well as apical constriction facilitating midline neural plate shaping. Furthermore, we summarize recent studies that shed light on how the interplay of signaling pathways and cell biomechanics modulate neural plate internalization. In addition, we discuss how adhesion-dependent cell-cell contact appears to be a critical component during midline cell convergence and surface cell contraction via cell-cell mechanical coupling. We envision that more detailed high-resolution quantitative data at both cell and tissue levels will be required to properly model the mechanisms of vertebrate neural plate internalization with the hope of preventing human neural tube defects.

KEY WORDS: *neurulation, morphogenesis, collective cell dynamics, cell contractility*

Introduction

The internalization of the early vertebrate neural ectoderm, a process otherwise known as neurulation, is a fundamental step leading to neural tube formation. Defects in the closure of the neural tube result in a series of devastating congenital disruptions, collectively known as neural tube defects (NTDs) (reviewed by Copp *et al*., 2003; Greene and Copp, 2014). While the general molecular principles of neural induction and patterning has been significantly investigated over the past years (reviewed by Weinstein and Hemmati-Brivanlou, 2013), our understanding of the cellular and biomechanical mechanisms driving vertebrate neurulation and human NTDs remains still rudimentary. This is partially because the mechanisms that shape the neural plate into a neural tube are complex and vary significantly among main vertebrate animal groups as well as along the anterior-posterior axis of the same organism (Nikolopoulou *et al*., 2017). In amniote embryos including mammals and birds, the neural plate is a single polarized epithelium with well-defined apical junctional components at the most apical surface while underlain by extracellular matrix at the most basal side (Schoenwolf and Franks, 1984). In anamniote embryos on the other hand, the organization of the neural plate is more variable and can be a single-layered epithelial structure as in newts (Brun and Garson, 1983), a bi-layered epithelium like in frogs (Schroeder, 1970), or a rather more complex layered structure with hybid features of both immature epithelium and mesenchyme as in teleost fish (Clarke, 2009; Araya *et al*., 2019). Although these early differences in cell and tissue cyto-architecture are commonly thought to influence subsequent steps of neurulation (i.e. dorsal folding, where the central canal is directly formed by an inward movement of a sheet of cells versus cavitation, where the central canal is only generated after the formation of a transient solid structure with no lumen), evidence shows that initial stages of neural plate morphogenesis are characterized by a series of

Abbreviations used in this paper: AC, apical constriction; AJ, adherens junction; CE, convergence and extension; DLHP, dorso-lateral hinge point; Dvl, disheveled; E-cadherin, epithelial cadherin; Fz, frizzled; Fmi/celsr, flamingo/celsr; INM, interkinetic nuclear migration; MHP, medial hinge point; MLC, myosin light chain; N-cadherin, neural cadherin; NTD, neural tube defect; PCP, planar cell polarity pathway; Pk, prickle; RhoGEF, Rho guanine nucleotide exchange factor; ROCK, Rho-associated coiled-coil protein kinase; Vangl/Stbm, van gogh/strabismus.

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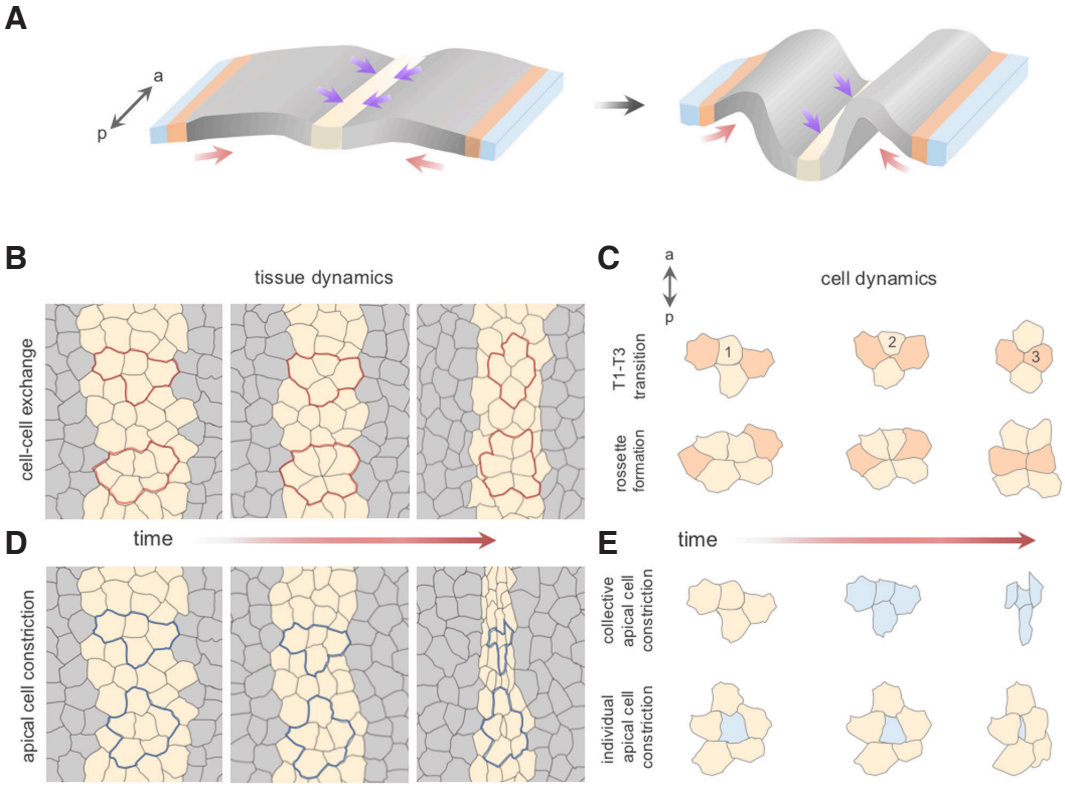

Fig. 1. Cell and tissue dynamics during neural plate internalization. (A) *Schematic representation of the vertebrate neural plate tissue dynamics during midline internalization. Grey color depicts lateral region of the neural plate while central yellow stripe indicates medial regions of the neural plate. Orange color depicts neural plate border while pale blue colors depict non-neural ectoderm. Red arrows indicate tissue movements, and purple arrows show midline apical cosntriction. (a) indicates anterior, while (p) indicates posterior.* **(B)** *Top view representation of midline neural plate cell dynamics during CE (cell-cell exchange). Encircle cells denotes cell behaviors in (C). In all panels anterior is up.* **(C)** *Top panels depict an example of T1-T3 transition. Here, orange cells intercalate between the yellow cells. This behavior is first driven by the shrinkage of the horizontal junction (1), then a transitory 4 ways configuration (2), following by a new junction along the a-p axis (3). Bottom panels depict an example of multi-cellular rosette formation. In both cases junction shrinking is driven by the polarized enrichment of Myosin-II.* **(D)** *Top view representation of midline neural plate cell dynamics during AC. Encircle cells denotes cell behaviors in (E).* **(E)** *Top panels depict collective AC events, while bottom panels depict individual AC event.*

common collective cell remodeling events at the developing dorsal midline (Colas and Schoenwolf, 2001; Wallingford and Harland, 2002; Williams *et al*., 2014; Butler and Wallingford, 2018; Araya *et al*., 2019). Moreover, while neural tube formation has been shown to be influenced by the interaction with adjacent tissues (Morita *et al*., 2012; Araya *et al*., 2014) and molecular cues (Ybot-Gonzalez *et al*., 2002; Araya *et al*., 2015), isolated tissue explants (Elul *et al*., 1997; Keller *et al*., 1992) as well as quantitative live imaging studies (Rolo *et al*., 2009; Nishimura *et al*., 2012; Galea *et al*., 2017) support the notion that early neural plate shaping depends largely on the activities of neural cells themselves. We therefore focus our discussion here on the intrinsic cellular and molecular events shaping the neural plate and leading towards internalization during early vertebrate neurulation. For more general reviews of vertebrate neurulation and NTDs, we refer readers to excellent reviews (Colas and Schoenwolf, 2001; Greene and Copp, 2014; Nikolopoulou *et al*., 2017).

One major step during early neural midline development is the orchestrated narrowing of a large body of neural plate cells towards dorsal midline territories (Fig. 1A). By this mechanism, the nascent neural ectoderm undergoes consistent reduction in width (convergence) while allowing the tissue to lengthen (extension) in the orthogonal anterior-posterior direction (Colas and Schoenwolf, 2001; Wallingford and Harland, 2002). Furthermore, evidence shows that the coordination of this process is central for midline tissue elongation in the whole embryo, as defects in this mechanism result in embryos with reduced body axis formation (Wallingford *et al*., 2006). While the identification of collective "convergence and extension" (CE) cell movements in neural progenitors have been widely indicated to be central during early vertebrate neurulation (Wallingford and Harland, 2002; Nikolopoulou *et al*., 2017), the cellular and molecular details of this process remains only partially explored. Analysis in both amniote and anamniote embryos has indicated that neural CE cell movements can be achieved by a heterogeneous set of cell behavior including single cell intercalation, orientated cell division, and neighbor exchange by junction remodeling (Colas and Schoenwolf, 2001; Nikolopoulou *et al*., 2017). Although the precise contribution of each cell behavior to the process of neural plate narrowing still await to be analyzed, genetic findings show that these collective cell movements are largely dependent on the non-canonical Wnt/Planar Cell Polarity (PCP) signaling pathway (Gray *et al*., 2011). During neurulation, depletion of PCP components results in embryos with abnormally short body axes, impaired neural plate internalization and NTDs (Wallingford and Harland 2002; Ciruna *et al*., 2006; Wang *et al*., 2006; McGreevy *et al*., 2015). Recent live imaging studies have revealed that during neurulation PCP components show asymmetric subcellular distribution in neural plate cells (Nishimura *et al*., 2012; Williams *et al*., 2014; Butler and Wallingford, 2018) and alter the biomechanical properties of the tissue during neurulation (Galea *et al*., 2018). However, how asymmetric distribution of PCP effectors is translated to cell-cell remodeling and biomechanical events during midline neural plate morphogenesis is still poorly understood.

Another major regulator of vertebrate neural plate internaliza-

tion is the collective cell shape changes of neural progenitor cells within the midline itself. In amniote and amphibian embryos, this collective cell shape change is thought to facilitate the formation of a neural groove, a transient embryonic structure required to initiate neural plate bending and tissue closure (Colas and Schoenwolf, 2001, Fig. 1A). While other embryos such as teleost fish form a neural tube in the absence of an obvious morphological neural groove, midline tissue constriction is still a key step that initiates an organized transient multicellular neural keel structure (Araya *et al.*, 2019). At the cell level, tissue internalization is chiefly achieved by apical cell surface reduction followed by the concomitant elongation along its apical-basal axis as cells deepen into the tissue (Sawyer *et al.*, 2010; Martin and Goldstein, 2014). The mechanism of apical cell constriction has been intensely investigated in many developing contexts (Sawyer *et al.*, 2010) and is thought to play a central role during early neurulation as embryos with defective mechanisms of apical constriction show impaired tissue closure and NTDs (Haigo *et al.*, 2003; Nishimura and Takeichi, 2008). Genetic analysis in both invertebrate and vertebrate systems has shown that the machinery responsible for apical cell constriction is largely powered by mechanical forces of the Actin filaments along with the Actin-binding motor protein non-muscle Myosin-II (the so called Actomyosin complex, Martin and Goldstein, 2014). Actomyosin complex dynamics and its associated partners are thought to confer cortical tensile properties to cells allowing irreversible surface constriction in localized cell junction surface domains (Martin *et al.*, 2009; Nishimura *et al.*, 2012). How midline convergent cell movements and apical cell constriction events are coordinated during vertebrate neural plate internalization is not well understood. However, it is clear that mechanical coupling between adjacent cells appears as key regulators of collective midline cell-cell morphogenesis (Morita *et al.*, 2010; Hong and Brewster, 2006). During neurulation, the expression of classical type-I cadherin member N-cadherin has been indicated to play a major role in mechanical coupling during early neural plate development (Radice *et al.*, 1997; Hong and Brewster, 2006; Nandadasa *et al.*, 2009; Morita *et al.*, 2010; Araya *et al.*, 2019). However, how cell-cell adhesion systems integrate and transmit both biochemical and biomechanical information during neural plate internalization is not well understood.

Midline cell dynamics during neural plate narrowing

The cellular basis of vertebrate neural tube formation has been intensively investigated as a means to understand how organ morphogenesis emerges from collective cell deformations (reviewed in Colas and Schoenwolf, 2001; Nikolopoulou *et al.*, 2017). Previous histological and ultra-structural studies in mammalian (Smith *et al.*, 1994), avian (Schoenwolf and Franks, 1984) and amphibian embryos (Schroeder, 1970) have indicated that vertebrate neurulation begins with the narrowing of a flat sheet of dorsal neural ectodermal cells (Colas and Schoenwolf, 2001, Fig. 1A). Moreover, these studied also revealed that tissue narrowing is concomitantly accompanied by the formation of a neural groove at the developing dorsal midline (Fig. 1A). While this dramatic tissue deformation has been normally associated to changes in cell morphology (i.e. cuboidal to bottle-shaped change following by cell elongation, Schroeder, 1970; Schoenwolf and Franks, 1984), these serial cross-section studies also reported a consistent reduction in the number of neural progenitor cells per section during successive stages of neural plate narrowing (Jacobson, 1984). Through the advance of early time-lapse video microscopy, cell labelling and tissue explants of frog embryos, the analysis of collective cell motility offered a better understanding of the cell dynamics during tissue shaping (Keller *et al.*, 1984; Shih and Keller, 1992). In fact, pioneer work from Keller and colleagues (1992) in isolated *Xenopus laevis* tissues of prospective axial chorda-mesoderm (commonly known as Keller explants) established the foundations for the analysis of CE mediated by collective cell intercalation (Keller *et al.*, 1984; Shih and Keller, 1992). Further studies refined this idea by showing that CE in vertebrate mesodermal tissues is partially achieved by the coordinated activity of medio-laterally orientated cell protrusions allowing cells to exert traction on their neighbors (Davidson and Keller, 1999). Although this mode of CE, sometimes known as cell crawling events (Shindo, 2018) has been instrumental for most mesenchymal tissue-type where cells lack clear apical-basal polarity and well-established cell-cell adhesion junctions, it does not faithfully describe the cell intercalation process of polarized epithelia. By the mid 2000's studies of germband extension in *Drosophila melanogaster* embryos demonstrated that collective CE cell movements during epithelial morphogenesis depends on polarized cell-cell junction behavior rather than cell crawling events (Bertet *et al.*, 2004; Blankenship *et al.*, 2006). CE in polarized tissues is thought to occur via the coordinated shrinking of two adjacent cell junctions (T1 transition), followed by an intermediate step establishing a common junction shared by four cells (T2 transition) and the subsequent construction of a new junction perpendicular to the previous T1junction (T1-T3 transition, Bertet *et al.*, 2004, Fig. 1C). In gastrulating flies as well as in other epithelial tissues, the T1 transition is normally orientated along the perpendicular axis of the tissue while the T3 transition is generally resolved along the major tissue axis (Bertet *et al.*, 2004). CE driven junction remodeling is powered by myosin-II, which accumulates in a polarized fashion allowing junction shrinking during T1 transitions (Bertet *et al.*, 2004). Junctional shrinking events in polarized epithelia may involve either two adjacent neighbor cells (T1 transition) or rather several cells simultaneously in order to generate transient supracellular complexes (i.e. rosette structure, Blankenship *et al.*, 2006, Fig. 1C). Similar to the T1 transition, transient rosette structures resolve along the plane of the major tissue's axis, allowing efficient neighbors exchange while maintaining tissue integrity during tissue morphogenesis (Blankenship *et al.*, 2006, Fig. 1C).

Midline domains of the developing neural plate have been shown to be particularly critical for effective CE cell movements (Wallingford and Harland, 2002; Ybot-Gonzalez *et al.*, 2002; Wang *et al.*, 2006; Ossipova *et al.*, 2015). While previous studies have suggested that vertebrate neural plate dorsal convergence relies largely on 2D cell crawling events, typically found in mesenchyme tissues (Elul *et al.*, 1997; Ezin *et al.*, 2003), new quantitative *en face* imaging approaches have provided a more complex 3D view of this process (Nishimura *et al.*, 2012; Williams *et al.*, 2014; Butler and Wallingford, 2018; Araya *et al.*, 2019). Collectively, these works have shown that CE in the vertebrate neural ectoderm is often achieved by a combination of cell crawling and cell junction remodeling events. In avian embryos, analysis of midline neural plate cells show that CE is largely mediated by polarized junctional remodeling events involving both T1-T3 transition and transient rosette-like structures (Nishimura *et al.*, 2012, Fig. 1 B-C). More recently, a sophisticated

ex-utero imaging analysis in the developing mouse embryo has revealed that CE in the midline neural plate is achieved by rather different mechanisms across the superficial-deep axis of the tissue (Williams *et al.*, 2014, Fig. 2A). At apical levels, midline convergence is organized through cell junction remodeling events including both T1-T3 transition and rosette supracellular structures, however, basally progenitors use preferentially mediolaterally-orientated cell protrusions (William *et al.*, 2014, Fig. 2A). While the murine neural plate model for CE seems to combine basal cell protrusions and apical junction remodeling within the same monolayer structure (Fig. 2A), these two cell mechanisms for CE appear to be restricted to specific tissue layers in anamniote vertebrates. In *Xenopus laevis*, the neural plate is organized as a bi-layered tissue structure composed by a highly polarized superficial layer and a non-polarized deep layer (Shoerder, 1970; Elul *et al.*, 1997; Ezin *et al.*, 2003, Fig. 2B). Previous studies in this model indicated that medial deep neural plate cells (notoplate) influence neural plate CE by using midline-orientated monopolar protrusions (Ezin *et al.*, 2003, Fig. 2B) and upon removal of midline structures (notoplate/notochord) cell protrusions of deep neural plate progenitors become bipolar (Ezin *et al.*, 2003). While cell protrusion activity seems to be confined to deep neural cells, new live imaging studies in the superficial neural plate layer show that midline CE is achieved via polarized junction remodeling events (Butler and Wallingford, 2018). In this layer, neural plate cells were characterized to undergo midline CE cell remodeling by using T1-T3 transition along medio-lateral/anterior-posterior axis as well by means of transient multicellular rosette structures (Butler and Wallingford, 2018). However, how these two modes of collective CE movements are coordinated in space and time to achieve neural midline development is currently unknown. Three-dimensional cell-cell rearrangements during tissue CE has only recently began to be explore in gastrulating *Drosophila* embryos. Previous studies in the fly have shown that apical junction shrinking dynamics is required during germband extension, however new findings now reveal that basolateral protrusion actively cooperate during epithelial morphogenesis (Sun *et al.*, 2017). Further findings in this study also show that these polarized basal protrusion events proceed faster than apical junction remodeling during supracellular rosette formation (Sun *et al.*, 2017). Similar to other vertebrate embryos, in teleost fish midline convergent cell movements also play a major role during neural plate midline morphogenesis (Ciruna *et al.*, 2006; Tawk *et al.*, 2007). Of interest, teleost embryos begin neurulation in prospective brain and anterior spinal cord regions with an unconventional multi-layered neural plate structure (Tawk *et al.*, 2007, Fig. 2C) and mature markers of apical polarity (i.e. aPKC, Pard3, Pard6, ZO-1) become only firmly established at advanced stages of neurulation (i.e. neural keel-rod transition, Tawk *et al.*, 2007). Interestingly, analysis of cell behavior in the zebrafish show that neural plate cells show little medio-lateral intercalation during dorsal midline behavior (Araya *et al.*, 2019). Instead, they appear to move rather as a cohesive sheet. Current evidence suggests that dorsal midline morpho-

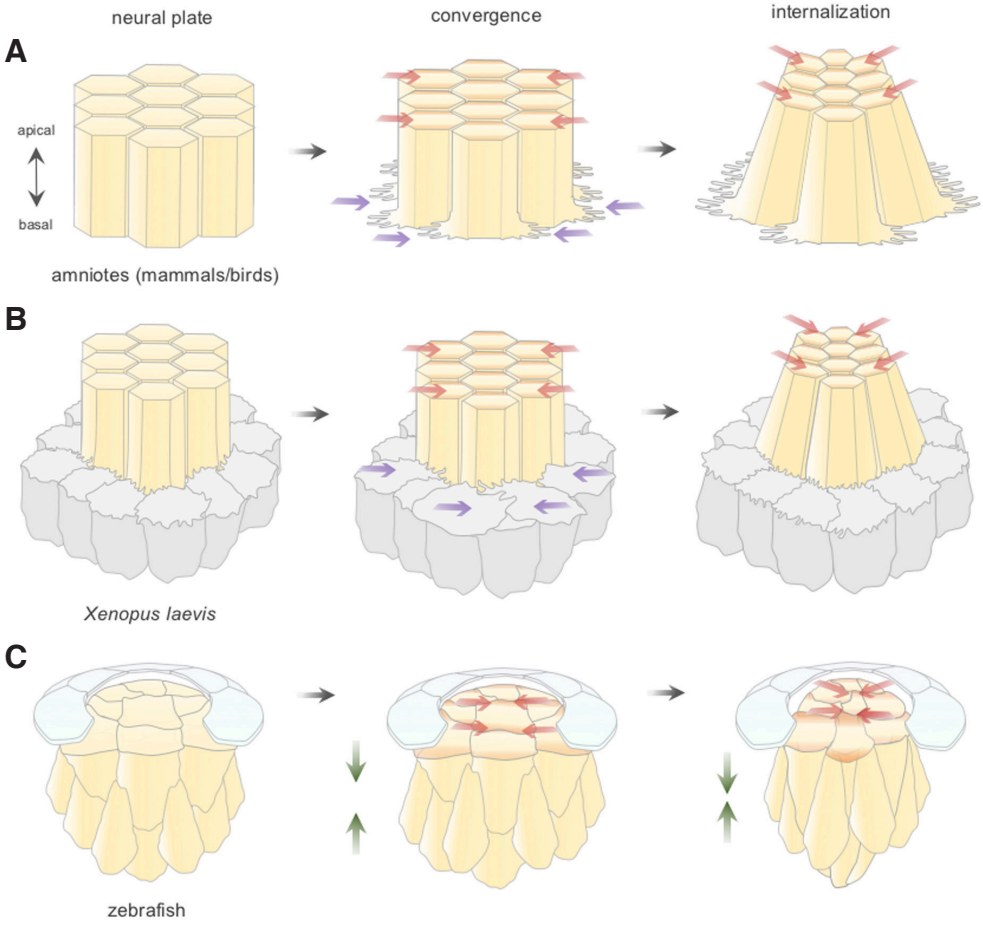

Fig. 2. Vertebrate neural plate cell organization during midline internalization. **(A)** *The amniote neural plate is composed of a single epithelial monolayer. Middle panel: neural plate convergence is driven by both apical junctional remodeling (red arrows) and basal cell protrusion (purple arrows). Right panel: cell and tissue internalization is thought to occurs by apical cell constriction (red arrows).* **(B)** *Left panel: in* Xenopus laevis*, the neural plate is a bi-layered structure composed of a superficial epithelial layer (yellow cells) and basal non-polarized mesenchyme cells (gray cells). Middle panel: in frogs, neural plate convergence occurs by both apical cell junction dynamics (red arrows) and basal midline-orientated monopolar protrusions (purple arrows).* **(C)** *Left panel: in zebrafish embryos, the neural plate cells is a multilayered tissue (yellow cells). Top pale blue cells depict the protective enveloping layer. Middle panel: neural plate cell converge in the zebrafish occurs by apical surface cell remodeling (red arrows) and radial intercalation across the superficial-deep axis of the tissue (green arrows). Right panel: in the zebrafish, cell internalization is driven by cell surface reduction (red arrows).*

genesis in the zebrafish neural plate occurs by a combination of radial cell intercalation (Hong and Brewster, 2006) and by direct cell surface remodeling events within midline domains (Araya *et al*., 2019). At the deep surface of the zebrafish neural plate, a tight association with the subjacent mesoderm (Araya *et al*., 2014) and the extracellular matrix (Araya *et al*., 2016) appears to be required to coordinate cell movements in the plate and to couple these to the underlying mesoderm. Whether basal neural plate cells use cell crawling based mechanism to achieve zebrafish neural plate midline convergence will need further investigations.

Molecular mechanisms of midline neural plate cell convergence

Depletion of several PCP pathway members including Van Gogh/Strabismus (*vangl*/*stbm*) (*vangl2* in vertebrates, Ciruna *et al*., 2006; Wang *et al*., 2006; Tawk *et al*., 2007), Disheveled (*dvl*, Hamblet *et al*., 2002), Scribble (*scrb1*, Murdoch *et al*., 2003), Frizzled (*fz*, Wang *et al*., 2006), Prickle (*pk*, Lu *et al*., 2004), Diego (*dgo*, Simons *et al*., 2005), and Flamingo/Celsr (*fmi/celsr*, Nishimura *et al*., 2012) results in defective neural plate convergence and severe NTDs (reviewed in Wallingford, 2006). Central for PCP signaling transduction is the ability to establish subcellular asymmetric localization of its core components while keeping a global planar polarity read out at the tissue level (reviewed by Gray *et al*., 2011). Moreover, mutant analysis and live imaging studies in both invertebrate and vertebrate organisms have shown that during PCP activity *pk* and *vangl/stbm* components are restricted to one extreme of the cell, while *dvl* and *fz* become localized into the opposite extreme of the cell (Gray *et al*., 2011). In addition, these studies indicate that the atypical cadherin Fmi/Celsr1 is required to reinforce cell-cell adhesion during PCP signaling transduction (Gray *et al*., 2011). Recent live imaging studies have shown that PCP components also display distinctive spatial and temporal patterns of subcellular localization during vertebrate neural plate dynamics. In avian embryos for instance, PCP components including Celsr1, Dvl as well as Fz show an apically localized distribution but with a marked posterior subcellular orientation (Nishimura *et al*., 2012). In the bi-layered neural plate of *Xenopus* embryos on the other hand, the PCP members including Pk and Vangl2 are expressed within the superficial layer where they show an anterior asymmetric localization (Ossipova *et al*., 2015; Buttler and Wallingford, 2018). While the above studies have revealed PCP signaling at spatial cell resolution, the evidence of PCP activity at subcellular levels in other vertebrate is still far less conclusive. In the zebrafish, standard methods to detect endogenous levels of PCP protein have failed to show any clear subcellular asymmetric distribution at neural plate stages and only show a molecular bias at keel and rod stages (Ciruna *et al*., 2006).

The asymmetric localization of core PCP components in neural cells raises the question of how PCP signaling mediates midline cell and tissue dynamics during vertebrate neurulation. In recent years, several lines of evidence have suggested that the PCP pathway acts in concert with the increasingly well characterized cytoskeletal machinery to effect junction remodeling during tissue morphogenesis (Nishimura *et al*., 2012; McGreevy *et al*., 2015). During neurulation, recent studies in chick and frog models have indicated that the spatial asymmetry of PCP components is required for polarized activity of the actomyosin motor complex at cell-cell junctions (Nishimura *et al*., 2012; Ossipova *et al*., 2015; Butler *et al*., 2018). In chick embryos, PCP activity is thought to mediate myosin junction localization at apical/superficial levels of neural progenitors (Nishimura *et al*., 2012). In the avian neural plate cells, the activation of Dvl and Fz result in the recruitment of the Formin related-protein Daam1 that is able to bind and activate PDZ-RhoGEF (Nishimura *et al*., 2012). PDZ-RhoGEF in turns recruits Rho Kinase-dependent activity via RhoA to promotes myosin phosphorylation at anterior (mediolateral) cell surface junctions (Nishimura *et al*., 2012). In the mouse model however, PCP signaling appears to be functionally dissociated along the superficial-deep axis of the neural plate (Williams *et al*., 2014). In these embryos evidence indicates that *vangl2* is restricted to anterior-apical domains of cells and promotes apical cell-cell remodeling behavior while the PCP-related protein Inactive tyrosine-protein kinase 7 (*ptk7*) preferentially regulates medio-lateral cell intercalation at deep/basal levels (Williams *et al*., 2014). The asymmetric localization of PCP components within discrete cell compartments may well reflect differences in PCP activities at specific cell domains. Thus, while in avian neural plate PCP seems to signal through Celsr1/Fz/Dvl components (Nishimura *et al*., 2012), a similar functional complex has not been found in other vertebrate contexts. Instead, in *Xenopus laevis* Vangl2 and Pk in the neural plate show an apical anterior (medio-lateral) enrichment but fail to show Fz/Dvl expression at more posterior domains (Ossipova *et al*., 2015; Butler and Wallingford, 2018). Whether this asymmetry in core PCP pathway components reflects complementary feedback mechanisms of adjacent junctions or rather represent species-specific requirements for PCP activity will need further clarification.

Another key unresolved question is how myosin junctional accumulation relates to tissue-level changes during neural plate midline convergence. During chick neural plate bending, myosin is preferentially accumulated in a long cable-like supracellular structures across the width of the tissue (Nishimura *et al*., 2012). Moreover, these supracellular actomyosin cables are thought to coordinate cell-cell rearrangements during neural plate bending, as depletion of myosin phosphorylation activity at these stages results in defective neural tube closure (Nishimura *et al*., 2012). In the mouse neural plate however, apical myosin appears to be uniformly distributed around cells and without apparent supracellular organization (Williams *et al*., 2014) and only at more basal positions myosin displays a preferential medio-lateral orientation (Williams *et al*., 2014). In the frog neural plate however, myosin activity is largely accumulated in individual cells with a marked anterior bias but without clear cable-like organization (Butler and Wallingford, 2018). During zebrafish neurulation, myosin expression is also temporally correlated with the initial steps of neural plate internalization. At these stages, myosin has distinctive accumulation at the superficial surface close to the dorsal midline domain although some myosin accumulation can be also found at the basal surface of the neural plate (Araya *et al*., 2019). Moreover, myosin-II distribution in the zebrafish neural plate does not show any obvious evidence for multicellular mediolateral cables (Araya *et al*., 2019), suggesting that individual cell contractility may be more important in this system. Thus, whether the generation of supracellular actomyosin cables is a defining feature of vertebrate neurulation in higher organisms or rather a species-specific mechanism required for tissue bending will need further investigation. Supracellular actomyosin assemblies have been shown to play

important roles during animal tissue morphogenesis and evidence shows that supracellular actomyosin structures show higher tensile values compared to single actomysoin activity at cell-cell interfaces (Blankenship *et al*., 2006; Calzolari *et al*., 2014). During avian neurulation these actomyosin cables may confer higher contractile forces required for medio-lateral tissue bending (Nishimura *et al*., 2012). The need for higher tension values during tissue bending may well represent a counter-balance mechanism to antagonize anterior-posterior forces required during neural tube closure (Zhou *et al*., 2009). On the other hand, medio-lateral actomyosin cables may also work as effective mechanisms limiting cell mixing during midline cell intercalation (Calzolari *et al*., 2014). Moreover, actomyosin cables have been recently indicated to directly influence cell division orientation during epithelial morphogenesis by the alignment of cell division machinery perpendicular to the cable (Scarpa *et al*., 2018). Interestingly, actomyosin cables in the chick neural plate are detected by 8 HH stage (Nishimura *et al*., 2012), a period characterized by oriented cell division behavior along the anterior-posterior axis (Sausedo *et al*., 1997).

Coordinated cell shape changes during vertebrate neural plate internalization

A defining feature in tissue internalization is the ability to organize collective cell deformation at precisely defined positions within developing organs. During vertebrate neurulation, neural plate cells undergo coordinated changes in morphology within the nascent dorsal midline in order to initiate efficient cell and tissue deformation (Haigo *et al*., 2003; Nishimura and Takeichi, 2008). In most vertebrates this collective cell shape change drives the formation of a midline furrow or neural groove (Colas and Schoenwolf, 2001). Ultra-structural analysis as well as live imaging studies have shown that these cell shape changes are by and large driven by a cell surface reduction mechanism, known as apical constriction (AC) (Sawyer *et al*., 2010). AC is a fairly well characterized cell mechanism in many epithelial contexts, whereby cells undergo progressive reduction of their apical surface while often expanding their basal domains (i.e. wedge-shape cell configuration, Martin and Goldstein, 2014, Fig. 2A). In addition, quantitative 3D live imaging studies have indicated that cell surface reduction during AC is accompanied by orthogonal cell elongation and therefore implying a certain degree of volume conservation principle (reviewed by Martin and Goldstein., 2014). While AC driving cell shape change has been shown to play a central role during vertebrate neural tube formation (Nishimura and Takeichi, 2008; Williams *et al*., 2014), how this cell mechanism is spatially coordinated with other neuroectodermal cell behavior is not entirely understood. In amniote neurulation, localized apical cell constriction events result in the formation of both a single medial hinge point (MHP) and then a pair of dorso-lateral hinge points (DLHP) at either side of the developing neural plate midline (Colas and Schoenwolf, 2001). In a cross-section view, the early amniote neuroepithelium has a pseudostratified tissue-structure composed of columnar cells with nuclei at different apical-basal levels (Sauer, 1935). These nuclei positions (driven by interkinetic nuclear migration, INM) represent neural cells at different stages of their cell cycle (Baye and Link, 2007). Thus, in the early amniote neural plate the G1, G2, and S-phases are largely confined at basal surface, while mitosis is largely devoted to occur towards the apical surface (Baye and Link, 2007). In the chick and the mouse, the initial stages of MHP formation are largely associated with slower cell cycle lengths of neural progenitors (McShane *et al*., 2015), and where most nuclei (at least 70%) are basally-located (Smith and Schoenwolf, 1987). Thus, while INM cell behavior favors the formation of wedge-shape cell configuration at the ventromedial positions of the neural plate, the molecular details of this coordination still need further investigation (reviewed by Eom *et al*., 2013). In the bi-layered neural plate of *Xenopus* embryos, tissue internalization is initiated by the apical cell constriction events of medially-located neuroepithelial cells of the polarized superficial layer (Schroeder, 1970, Fig. 2B). Through this mechanism, superficial neural plate cells undergo wedge-shaped changes in order to generate a shallow depression or neural groove between the neural ectoderm and the above vitelline membrane (Schroeder, 1970). Subsequently, this neural groove deepens towards more inward positions while concomitantly a pair of dorsal folds emerge at both sides of the midline (Schroeder, 1970). By these stages, the frog neural plate undergoes extensive radial intercalation between its superficial and deep layers as cells become elongated in shape (Davidson and Keller, 1999). Although superficial cells appear to initiate neural groove formation in the frog neural plate whether deep cells influence and refine surface cell dynamics needs further characterization. In teleost fish, the mechanisms of midline tissue internalization slightly differ from those described for amniote and amphibian embryos. In the anterior multi-layered zebrafish neural plate, surface medial cells move inwards to generate a transient multi-cellular neural keel without the need of a conventional neural groove structure (Tawk *et al*., 2007; Araya *et al*., 2019). Cell behavior analysis at these stages reveal that this medial inward cell behavior is also controlled by cell surface reduction mechanisms (Araya *et al*., 2019, Fig. 2C). While this superficial cell constriction event is closely comparable to the ones depicted for other vertebrate classes, zebrafish neural plate cells do not experience wedge-shaped changes. Instead, they undergo consistent elongation along the superficial-deep axis of the developing plate as they converge towards the midline before internalization (Hong and Brewster, 2006; Clarke, 2009, Araya *et al.,* 2019, Fig. 2C). In addition, live imaging analysis suggests that this cell shape remodeling and internalization mainly operates on a cell-by-cell basis rather than a cell collective event (Araya *et al*., 2019). Asynchronous cell-autonomous apical constriction events have been also found during *Xenopus laevis* neural plate internalization although its significance is still unknown (Christodoulou and Skourides, 2015, Fig. 1 D-E). While not fully understood, the preference of individual cell ingression over collective cell invagination during anamiote neural plate internalization may have evolved as an efficient mechanism to construct internal organs while preserving tissue surface integrity in highly-spherical embryos.

Molecular control of cell surface constriction dynamics during neural plate internalization

While apical constriction leading to neural plate bending or cell internalization has been widely documented during vertebrate neurulation, our current understanding of the likely molecular basis of this cell behavior has been inferred from the analysis of epithelial morphogenesis in invertebrate organisms (reviewed by Martin and Goldstein, 2014). During *Drosophila* gastrulation, apical cell constriction events drive the internalization of a stripe of

ventral-medial located cells in order to generate internal mesoderm tissues (Martin *et al*., 2009). In addition, *in vivo* analysis in this embryo has shown that apical cell surface reduction is mediated by the contractile activity of the actomyosin cytoskeletal apparatus (Martin *et al*., 2009). Genetic and cell biology studies in *Drosophila* have further shown that myosin-dependent apical cell constriction activity is partially orchestrated by Rho-Guanine Exchange Factor (Rho-GEF) regulating the Rho family of small GTPases member Rho-A. Rho-A in turns stimulates its downstream effector Rho-associated coiled-coil protein kinase (ROCK) to phosphorylates Myosin light chains (MLC) (reviewed in Vicente-Manzanares *et al*., 2009). Finally, MLC activation drives the association of non-muscle myosin-II with actin filaments within apical junctional belt regions of epithelial cells to efficiently drive surface constriction (Martin *et al*., 2009; Sawyer *et al*., 2010; Martin and Goldstein, 2014). In addition, sophisticated live imaging analysis show this actomyosin cell deformation event occurs by progressive steps of apical medial coalescence of myosin-II during the constriction phases followed by cortical maintenance during stabilization phases (Martin *et al*., 2009).

While many key players of the myosin-II regulation including Rho-GEF (Nishimura *et al*., 2012; Itoh *et al*., 2014), Rho (Itoh *et al*., 2014), ROCK (Nishimura and Takeichi, 2008), and myosin-II itself (Rolo *et al*., 2009; Araya *et al*., 2019) have been already identified to be central during early neurulation, their precise spatial and temporal roles during vertebrate neural plate remodeling surface remain still poorly explored. How myosin dynamics is regulated during neurulation is of particular interest as previous studies have led to the predominant view that an actomyosin network assumes a rather circumferential "cable" configuration around the apical cell cortex (Burnside, 1971). In this configuration, apical constriction is thought to occur via a rather progressive purse-string contraction (Haigo *et al*., 2003; Hildebrand *et al*., 2005). Additional support to this model comes from the central role the F-actin binding Shroom3 during vertebrate neurulation (Haigo *et al*., 2003; Hildebrand *et al*., 2005; Nishimura and Takeichi, 2008). In mouse and chick embryos, Shroom3 regulates ROCK activity at adherens junctions to organize circumferential actomyosin cables around the apical domains of neural plate cells (Hildebrand, 2005; Nishimura and Takeichi, 2008). While the molecular details of how Shroom3 regulates myosin ROCK-dependent activity to adherent junctions are still under investigation (McGreevy *et al*., 2015), the current view is that this activity is independent of Rho signaling (Nishimura and Takeichi, 2008). Lastly, an additional layer of complexity for Shroom3 function is related to its key function in microtubule assembly during cell elongation (Haigo *et al*., 2003) and recent evidence suggest that parallel arrays of microtubules are required to counterbalance compressive forces from actomysoin dynamics (Singh *et al*., 2018).

Recently it has been proposed that neural plate cell contractility may not proceed via smooth progressive changes but rather via oscillatory steps of pulsatile cell contractions. The study by Christodoulou and Skourides (2015) has shown that during early frog neurulation, neural plate cells undergo cell-autonomous and asynchronous apical cell constriction events (i.e. largely concentrated in prospective hinge points). Authors of this work also show that these cell deformation events are preceded by cell autonomous activity of Ca^{2+} and medial concentration of actin dynamic at the apical-medial cell surface (Christodoulou and Skourides, 2015). Additionally, the recent study by Butler and Wallingford (2018) has neatly shown evidence that a pulsed myosin-II behavior drives polarized cell shrinking dynamics during medial neural plate morphogenesis. Since pulsatile contractility operates in other morphogenetic examples of apical constriction (Martin *et al.,* 2009 this may be a conserved mechanism underlying divergent tissue deformations.

Coordinating cell behavior during midline neural plate morphogenesis

Previous studies have suggested that specific aspects of neural plate shaping may occur independently (Copp *et al*., 2003; Survillan-Brown and Goldstein, 2012). This view is partially supported by the fact the abrogation of convergent extension cell behavior mostly results in craniorachischisis (a severe clinical condition that result in the defective closure at brain and spinal cord regions, Green and Copp, 2014), while impaired apical contraction normally result in cranial NTDs (i.e. excencephaly, Copp *et al*., 2003). Alternatively, experimental observations and computational modelling has suggested that the interaction of these two collective cell dynamics is both necessary and additive to shape the vertebrate neural plate (Jacobson, 1984; Nielsen *et al*., 2020). In support of this a recent work in the mouse has shown that both Sroom3 and the PCP pathway (*vangl2*) genetically interact to enhance vertebrate NTD phenotype (McGreevy *et al*., 2015). While, the specific contribution of CE and AC during vertebrate neural plate bending still awaits for further clarification it may be these two potentially alternative scenarios are not mutually exclusive. On one side CE cell movements and AC events might occur at the same time but in spatially segregated regions of the neural plate. By this notion, AC might occur predominantly within midline regions while CE may operate in adjacent areas along the midline domains. In fact, CE is often found to occurs widely across the neural plate (Williams *et al*., 2014; Butler and Wallingford, 2018). On the other hand, CE and AC can still occur both within the midline region but in two separated times. Here, CE cell dynamics might control first midline cell displacement and neighbor exchange dynamics and subsequently AC facilitates cell and tissue internalisation at the midline itself. Supporting this idea is the fact that CE is normally achieved without apparent change in relative surface apical cell area (Butler and Wallingford, 2018, Fig. 3).

Role of cell-cell contacts in neural plate internalization

Collective cell dynamics largely depends on cell-cell adhesion dependent contact (Takeichi, 2014). Intercellular adhesion allows mechanical coupling between cells while allowing to integrate and respond to actomyosin contractile activities via cell shape deformation (Martin *et al*., 2010). The vertebrate neuroepithelium expresses all major components of cell-cell junctions typically found in epithelia including the tight junctions, desmosomes, and the adherens junctions (AJs) (reviewed by Eom *et al*., 2013). However, the AJs system at the cell's apical zonula adherens has been found to be particularly critical during early neural tube morphogenesis (Eom *et al*., 2013). A major components of the AJs system is the calcium-dependent transmembrane Cadherins receptors (Nishimura and Takeichi, 2008). Cadherins are single transmembrane proteins that consist of five extracellular domains (containing Ca^{2+} binding sites), a single transmembrane domain,

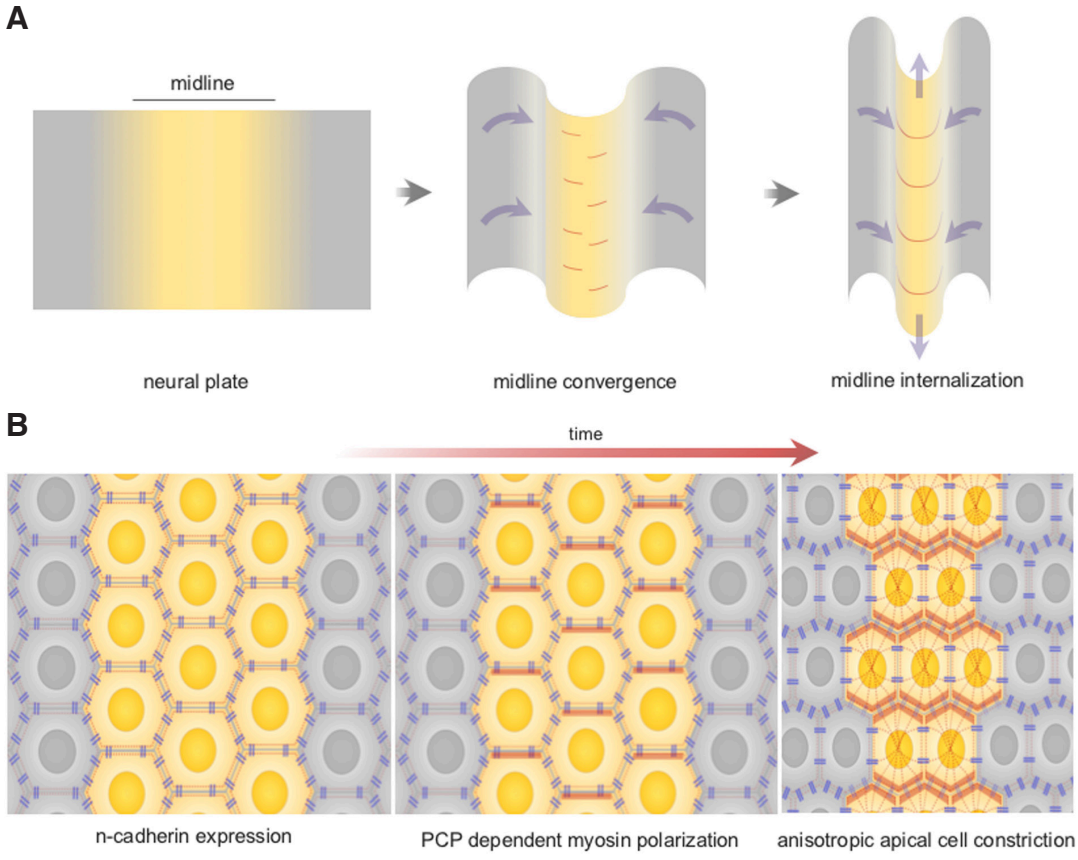

Fig. 3. Cell-cell adhesion and Myosin-II contractile activity organize vertebrate neural plate internalization. (A) *Schematic representation of the main stages of vertebrate neural plate internalization. Midline region is depicted as central yellow band, purple arrows represent tissue movements, and red lines indicate midline Myosin-II organization.* **(B)** *Diagrams showing the cellular and molecular events depicted in (A). Left panel: following neural plate induction, cells begin to express high levels of the cell-cell adhesion molecule N-cadherin (blue). Middle panel: the asymmetric expression of non-canonical Wnt/PCP signaling is thought to promotes cell-cell exchange throughout the anisotropic activity of Myosin-II (red). Right panel: N-cadherin further coordinates the activity of both cortical (red bars) and apical contractile Myosin-II network (central red dotted lines) to facilitate anisotropic cell surface reduction and tissue inward. In all panels, anterior is up.*

and a cytoplasmic tail, which anchors to the cell's cytoskeleton via binding of p120-catenin, and β-catenin (Hirano and Takeichi, 2012). β-catenin in turn binds to α-catenin to link F-actin microfilaments (Takeichi, 2014). Intercellular adhesion mediated by Cadherins is thought to occur by homophilic contact throughout their extracellular domains (trans-configuration) while they can also interact in *cis* with other monomers on the same plasma membrane (cis-configuration) (Hirano and Takeichi, 2012). Following neural induction, the vertebrate neural ectoderm begins to express high levels of the classical type-I N-Cadherin (also known as neural cadherin or Cdh2) at the expense of E-Cadherin (epithelial cadherin or Cdh1) on the cell's apical domain (Hatta and Takeichi, 1986; Dady *et al*., 2012). While the significance of Cadherin switching is not entirely understood, the temporal appearance of N-Cadherin in the neural plate is closely correlated with major tissue midline remodeling events including dorsal convergence and internalization (Dady *et al*., 2012, Fig. 3A). In addition, genetic studies have revealed that lack of N-Cadherin results in abnormal neural plate morphogenesis and NTDs (Hong and Brewster, 2006; Nandadasa *et al*., 2009, Araya *et al*., 2019).

How N-Cadherin drives neural plate morphogenesis is not well understood. On the one hand, we favor the view that the appearance of N-Cadherin may confer higher migratory properties to neural plate cells as they move towards the dorsal midline (Fig. 3B). Similarly to other biological contexts, E- to N-cadherin switching it is likely to impact AJs organization as well as their biochemical properties (Takeichi, 2014). Whereas in most stable epithelial tissues E-Cadherin is largely organized in linear AJs normally associated with bundles of actin filaments running in parallel to the plasma membrane (i.e. circumferential actin belts of zonula adherens), N-cadherin expression is typically organized as puncta AJs and where actin filaments perpendicularly terminate at the cell membrane (Takeichi, 2014). N-cadherin puncta organization is normally found in mesenchyme tissue-types in which cells have high motility and neighbor interactions (Charrasse *et al*., 2002). In addition, N-Cadherin has been also localized throughout the plasma membrane where it thought to mediates cell migration (Charrasse *et al*., 2002; Hong and Brewster, 2006). The intercellular mechanical coupling by N-cadherin activity during midline convergence may also be reinforced through the physical interaction with other cell-cell adhesion molecules including atypical protochaderins (Biswas *et al*., 2010), and the immunoglobulin member Nectin-2 (Morita *et al*., 2010). Although it is not yet understood how N-cadherin achieves collective cell motility, it is very likely that this cell behavior might involve major F-actin dynamics reorganization through Rho family GTPases activity (Charrasse *et al.,* 2002).

Apart from its role in mechanical cell coupling during collective midline cell motility, N-Cadherin may also play an active role in cell shape changes driving midline internalization. During morphogenesis, Cadherin contacts appear as critical components of cell shape deformation via control of junction cell remodeling (Martin *et al*., 2010; Takeichi, 2014; Ladoux and Mege, 2017). This Cadherin property is thought to occur via its antagonist role controlling actomyosin cortical tension at the contact cell junction. Work in developing epithelia have shown that cell junction dynamics are partially achieved by the asymmetric interplay between actomyosin activity promoting junction shrinking while AJs/Cadherins normally promote junction growth (Bertet *et al*., 2004; Rauzi *et al*., 2010).

Moreover, this asymmetric distribution of AJs/Cadherin at the plasma membrane not only strengthens the polarized actomyosin contractility within individual cells but also it helps to establish polarized cortical dynamics at the plane of the tissue (Bertet *et al*., 2004; Rauzi *et al*., 2010). During neurulation, the asymmetric enrichment of actomyosin activity by PCP signaling along the medio-lateral neural plate cell junctions is thought to confer cell and tissue orientation during midline bending (Nishimura *et al*., 2012, Fig. 3C). In turn, medio-lateral actomysoin enrichment may further result in a polarized redistribution of N-Cadherin towards anterior-posterior junction and therefore facilitating anisotropic apical constriction (Nishimura and Takeichi, 2012, Fig. 3B). While a potential mechanism of actomysoin dependent junction dynamics through N-Cadherin activity has been previously suggested (Nanadadasa *et al*., 2009; Morita *et al*., 2010; Araya *et al*., 2019), the molecular details of this interaction remains still unclear. Cadherin redistribution controlling junction remodeling may involve several mechanisms including recycling endocytosis (Levayer *et al*., 2011), PCP regulation (Dos-Santos Carvalho *et al*., 2020), as well as by modulation of actomyosin tension levels (Ladoux and Mege, 2017).

Conclusions and future directions

Vertebrate neurulation relies on collective cell rearrangements of a transient embryonic structure called the neural plate. Despite the fact that defective neural plate midline internalization may result in severe NTDs, the molecular and cellular details that govern this process remain still only partially characterized. In this review we have summarized past and recent studies that shed light on key aspects of cell-cell rearrangements and cell surface dynamics leading to neural plate internalization at the dorsal midline. We have discussed evidence that indicates that despite initial differences in tissue architecture among main vertebrate animal groups, neural plate morphogenesis can be largely defined by two main cell behaviors; namely dorsal convergent cell movements and collective cell shape changes at the tissue midline. In addition, we shed light on how the interplay of biochemical signaling pathways and conserved cellular mechanical properties facilitate midline neural plate morphogenesis. Moreover, we have shown evidence that suggests that Cadherin dependent cell-cell contact is as key modulator for midline neural groove formation.

Past and current research has characterized two general modes of collective CE during neurulation (i.e. crawling based on basal cell protrusions vs apical junction remodeling, Shindo, 2018). In this review we have argued that these two mechanisms may not be mutually exclusive but rather they appear to act in concert at distinctive sub-cellular domains within the superficial-deep axis of cells to efficiently drive neural plate morphogenesis (Williams *et al*., 2014; Butler and Wallingford, 2018). A common theme emerging from present findings is the pressing need for high-resolution imaging studies combining quantitative cell behavior analysis and reliable sub-cellular protein dynamics. The recent advent of novel *in utero* imaging studies in the mouse (Galea *et al*., 2017; Williams *et al*., 2014) along with well-established methodologies to study *in vivo* gene function in both amniote (Nishimura *et al*., 2012; Williams *et al*., 2014), and anamniote embryos (Butler and Wallingford, 2018; Araya *et al*., 2019) has led to a better understanding of *in vivo* cellular dynamics underlying vertebrate neurulation. In addition, the recent observation of the asymmetric distribution of PCP effectors in the neural plate has been key to relate sub-cellular signaling localization to global cell dynamic modulation (Nishimura *et al*., 2012; Butler and Wallingford, 2018). Although the details of how this asymmetry in non-canonical Wnt/PCP signaling is initially established and maintained in neural plate cells remains a major unresolved question in the field, it is likely to involve a developmentally regulated program (Nimomiya and Wilkbauer, 2004) along with biomechanical feed-back mechanisms to ensure morphogenesis progression (Ossipova *et al*., 2015). Further imaging studies of vertebrate neurulation would be necessary to adequately understand the conservation of CE and AC cell dynamics during neural plate midline development. While most of our current knowledge of neural plate morphogenesis has been gathered at hindbrain-anterior spinal cord regions it would be equally critical to study cell-cell rearrangements in other domains of the developing neuraxis, especially in anterior brain regions where NTDs/excencephaly arise (England *et al*., 2006). The recent characterization of the cellular and molecular mechanisms leading to posterior neural plate morphogenesis is a significant advance in the field (Galea *et al*., 2017; 2018). Although not discussed in this review, the development of quantitative imaging approaches to understand the contribution of adjacent tissues and cues to the process of vertebrate neurulation is equally important (Morita *et al*., 2012; Araya *et al*., 2014). Finally, the development of tools and methodologies to infer and in many cases to actually measure mechanical properties of cells and tissues will be critical to study the mechanical contribution during early neurulation. Particularly, the recent incorporation of optogenetic technology to control protein activity in time and space appears as an excellent approach to understand the specific biomechanical contribution of CE and AC to neural midline internalization (Krueger *et al*., 2018). Collectively, the use of extensive live imaging studies as well as the identification of mechanical properties of neural plate cells will result in more compelling computational simulations to model vertebrate neurulation at both cell and tissue levels (Jacobson, 1984; Nielsen *et al*., 2020).

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