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G-protein-coupled receptor signaling and neural tube closure defects

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Abstract

Disruption of the normal mechanisms that mediate neural tube closure can result in neural tube defects (NTDs) with devastating consequences in affected patients. With the advent of next-generation sequencing, we are increasingly detecting mutations in multiple genes in NTD cases. However, our ability to determine which of these genes contribute to the malformation is limited by our understanding of the pathways controlling neural tube closure. G-protein-coupled receptors (GPCRs) comprise the largest family of transmembrane receptors in humans and have been historically favored as drug targets. Recent studies implicate several GPCRs and downstream signaling pathways in neural tube development and closure. In this review, we will discuss our current understanding of GPCR signaling pathways in pathogenesis of NTDs. Notable examples include the orphan primary cilia-localized GPCR, Gpr161 that regulates the basal suppression machinery of sonic hedgehog pathway via activation of cAMP-protein kinase A signaling in the neural tube, and protease-activated receptors that are activated by a local network of membrane-tethered proteases during neural tube closure involving the surface ectoderm. Understanding the role of these GPCR-regulated pathways in neural tube development and closure is essential toward identification of underlying genetic causes to prevent NTDs.

Keywords

Neural tube defect; G-protein-coupled receptor; primary cilia; sonic hedgehog; Gpr161; protease-activated receptor; protein kinase A; cAMP

1. Introduction

Neural tube defects (NTDs) refer to structural birth defects of the brain and spinal cord, and constitute multiple diseases that affect neural tube development and closure. The incidence of NTDs ranges between 0.5–2 in 1000 live births (Botto et al., 1999). The incidence of NTDs has been drastically reduced by oral intake of folic acid (Greene and Copp, 2014). In addition, newer pre-natal surgical procedures to treat spina bifida seem promising in minimizing life-long disabilities (Adzick et al., 1998; Adzick et al., 2011). However, we urgently need to improve our understanding of the underlying causes of NTDs to prevent

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these debilitating disorders. G-protein-coupled receptors (GPCRs) constitute the most abundant transmembrane receptor family and have been extensively targeted for drug development (Dorsam and Gutkind, 2007; Lagerstrom and Schioth, 2008). In this review, we will discuss our current understanding of GPCR signaling pathways in pathogenesis of NTDs, with respect to recent developments in our understanding of GPCRs in sonic hedgehog signaling pathway and neural tube closure.

2. Neural tube development

The neural tube is formed from the dorsal ectoderm of the early embryo and gives rise to the brain and spinal cord. Development of the neural tube follows a precisely orchestrated series of events. As excellent reviews on this topic are available (Copp and Greene, 2010; Copp et al., 2003; Greene and Copp, 2014; Harris and Juriloff, 2007; Harris and Juriloff, 2010; Wallingford et al., 2013), we will briefly summarize the steps that could be relevant to GPCR signaling pathways in the context of neural tube development and closure.

2.1. Appearance of the neural plate

The neural plate is the first observable component of the central nervous system to emerge in the early mouse embryo around E7.5. As the neural plate is induced, it appears as a thickening of neuroepithelial cells that develops from the dorsal ectoderm (Figure 1A). The neural groove forms in the midline and neural folds at the lateral edges of the neural plate begin to rise and converge towards the dorsal midline to form the neural tube. (Figure 1B).

2.2. Bending of the neural plate and neural patterning

During neurulation, the edges of the neural plate elevate to form the neural folds. As the neural plate bends, the tips of the neural folds come together in the dorsal midline (Figure 1C). In the upper and mid spine, bending is greatest at the most medial and ventral aspects of the neural plate, called the median hinge point. In the mid and lower spine, bilateral dorsolateral hinge points are also prominent (Murdoch and Copp, 2010; Shum and Copp, 1996). Hinge points form as neuroepithelial cells transform from spindle-shaped to predominantly wedge-shaped with basal nuclei (Smith and Schoenwolf, 1987; Smith and Schoenwolf, 1988) (Figure 1D).

Neural patterning is one of the early events distinguishing ventral from dorsal regions of the neural tube. This patterning is established by counteracting gradients of long-range morphogens. While Wnt and BMP released from the roof plate of the neural tube and adjacent tissues promote dorsal identities, Shh release initially from the notochord, and later from the floor plate plays a critical role in ventral patterning (Ulloa and Briscoe, 2007). Sonic hedgehog (Shh) and bone morphogenetic protein (Bmp) signaling inhibits dorsolateral hinge point formation, whereas Bmp antagonism by Noggin induces dorsolateral bending (Ybot-Gonzalez et al., 2002; Ybot-Gonzalez et al., 2007). Bmp signaling can regulate apicobasal polarity pathway by modulating apical junctions in the neuroepithelial cells in a cell cycle-dependent manner, resulting in changes in cell and tissue shape (Eom et al., 2013; Eom et al., 2011; Eom et al., 2012). Lack of Shh signaling (for e. g. in *Shh* and *Smoothened* mutants) does not result in neural tube closure defects (Murdoch and Copp, 2010). In

contrast, increased Shh signaling results in NTDs accompanied by expansion of ventral domains into dorsal regions (ventralization), and inhibition of dorsolateral hinge points (Murdoch and Copp, 2010). In addition, both Shh and BMP signaling regulate the balance between neuroepithelial cell proliferation, apoptosis, and morphogenesis to form the typical morphology of the neural tube (Murdoch and Copp, 2010). The neural tube is of abnormal triangular morphology in some mutants with increased Shh signaling, such as in *Tulp3* (Patterson et al., 2009) and *PKA* mutants (Huang et al., 2002), with narrowed ventral and expanded dorsal regions. The thinning of the neuroepithelium in *Tulp3* mutants results from the misregulated expression of factors promoting or inhibiting neuronal differentiation in ventral and dorsal regions, respectively (Norman et al., 2009).

Shh signaling in neuroepithelial cells can regulate cell-cell and cell-extracellular matrix interactions by blocking the activation of β6 integrins at the apical pole independent of its canonical action on ventral patterning. Inactivation of β6 integrins causes upregulation of cadherins and apicobasal polarity genes. This results in reinforcement of adhesion between neuroepithelial cells, and local restriction of functional integrins to the basal side in contact with the matrix (Fournier-Thibault et al., 2009; Jarov et al., 2003; Testaz et al., 2001) (Figure 1E). The association of medial and dorsolateral hinge points with the notochord and surface ectoderm respectively is critical for generating bending forces and apposition.

2.3. Fusion of the neural folds and neural tube closure

Fusion initiates at primary closure points located at distinct anterior-posterior positions of the neural tube. As the tips of the neural folds converge towards the dorsal midline, the opposing folds fuse to form the neural tube (Figure 1C). By E8.5 in the mouse, the first point of neural tube closure initiates at the level of the hindbrain/cervical boundary, and is referred to as closure point 1 (Figure 1C). Closure spreads bidirectionally to seal the open region of the neural folds (as known as “neuropores”), with further *de novo* closure points initiating at the level of forebrain/midbrain boundary (“closure 2”) and extreme rostral end of the forebrain (“closure 3”). The neuropores gradually shorten and neural fold closure is completed by E9 in the anterior part of the brain, a few hours later at the hindbrain, and by E10.5 at the posterior neuropore. Closure is initiated by membrane protrusions resembling lamellipodia and filopodia emanating from surface ectodermal cells, followed by epithelial adhesion, and further remodeling to establish continuity (Pai et al., 2012; Rolo et al., 2016). Rho GTPases are critical in forming membrane protrusions; in particular, Cdc42 and Rac1 are necessary for predominance of filopodia during early neurulation, and membrane ruffle formation in late closure stages, respectively (Rolo et al., 2016). In addition, binding between GPI-linked EphrinA ligand and Eph A receptor might contribute to epithelial adhesion (Pai et al., 2012).

Planar cell polarity, a non-canonical Wnt-Frizzled signaling pathway has also been implicated in causing the most severe form of NTDs called craniorachischisis (open spinal cord and brain) in mice (Murdoch et al., 2014; Wallingford, 2006), and proteins in this pathway are associated with a range of NTDs in humans (Juriloff and Harris, 2012). The PCP pathway could regulate apical constriction along the medio-lateral axis of the neural tube, inducing polarized bending (see section on CELSRs) (Nishimura et al., 2012).

3. G-protein-coupled receptor (GPCR) signaling pathways

GPCRs are seven transmembrane receptors that couple to G proteins to activate second messengers in cellular signaling pathways (Katritch et al., 2013; Rosenbaum et al., 2009; Venkatakrishnan et al., 2013). Heterotrimeric G proteins consist of Gα, β and γ subunits (Hurowitz et al., 2000; Oldham and Hamm, 2008). Upon activation by ligands, GPCRs facilitate exchange of GDP for GTP on the Gα subunit resulting in dissociation of Gβ/Gγ dimers from the complex. Activated Gα proteins and dissociated Gβ/Gγ subunits regulate many downstream signaling pathways including activation ($G\alpha_s$) or inhibition ($G\alpha_i$) of adenylyl cyclases to regulate cAMP production, Ca^{2+} signaling ($G\alpha_q$-Gβ/Gγ) and activation of small GTPases such as Rho ($G\alpha_q$ and $G\alpha_{12/13}$) (Dorsam and Gutkind, 2007; Lagerstrom and Schioth, 2008). GPCRs constitute the largest superfamily of receptors in human genome and play critical roles in various physiological processes, such as vision, olfaction, and neurotransmission. GPCRs have been extensively targeted for drug development in multiple diseases with approximately 30–50% of current drugs acting on GPCRs (Garland, 2013; Overington et al., 2006). Importantly, recent improvements in crystallography, cryo electron microscopy, NMR, and computational modeling are providing fundamental insights into structures of GPCRs with respect to different functional states. These advances are transforming the potential for discovery of newer drugs based on modeling ligand binding pockets (Tautermann, 2014).

4. GPCRs and neural tube defects

4.1. Shh signaling pathway, primary cilia, and Gpr161

Basal suppression machinery of Shh signaling and primary cilia—The primary cilium is critical for Shh signaling in dorso-ventral neural tube patterning (Goetz and Anderson, 2010). Shh signaling culminates in processing of the bifunctional Gli transcription factors to generate Gli activators or repressors in a cilium-dependent manner. Addition of Shh results in removal of Patched (Ptch1), the Shh receptor from cilia, and accumulation of Smoothened (Smo), a frizzled family GPCR in the compartment. Smo determines activation of the pathway by promoting generation of Gli2 activator (Figure 2) (Mukhopadhyay and Rohatgi, 2014). In addition to Gli activator, Gli repressors (mainly Gli3R) are generated in a cilium-, protein kinase A- (PKA), and suppressor of fused (Sufu)-dependent manner upon proteolytic processing (Humke et al., 2010; Niewiadomski et al., 2014; Wang et al., 2000; Wen et al., 2010), referred to as the basal repression machinery of Shh signaling (Figure 2). However, the pathways determining cAMP-PKA signaling in the cillium are not well understood and are critical toward addressing the role of negative regulation of the pathway. In contrast to the activation arm of the pathway, repression of Shh signaling is fundamental for successful neural tube closure (Murdoch and Copp, 2010). Thus, elucidating pathways that control repression of Shh signaling are essential for uncovering mechanisms of NTDs.

Gpr161—Gpr161 is an orphan GPCR highly expressed in the neural tube during embryonic development (Matteson et al., 2008; Mukhopadhyay et al., 2013). Our recent work defines Gpr161 as a negative regulator of the Shh pathway by functioning in the basal suppression

machinery (Mukhopadhyay et al., 2013). Complete loss of *Gpr161* in embryos results in embryonic lethality by E10.5, and ventralization throughout the rostro-caudal extent of the neural tube. The mutant embryos also exhibit extensive craniofacial abnormalities, with open fore/mid brain regions, and caudal neural tube defects (Mukhopadhyay et al., 2013).

Gpr161 is trafficked to the primary cilia (Mukhopadhyay et al., 2013). *Gpr161* knockout embryos lack Gli3 repressor, and Gpr161 constitutively activates cAMP formation, suggesting that Gpr161 activates cyclic AMP-dependent PKA signaling in promoting Gli repressor formation in a primary cilia-dependent manner (Mukhopadhyay et al., 2013). The extensive craniofacial abnormalities, and caudal neural tube defects possibly result from increased Shh signaling in the neural tube, and/or adjacent mesenchyme, as noticed in varying severities in other mutants with increased Shh signaling (Hwang and Mukhopadhyay, 2015; Murdoch and Copp, 2010).

Following Shh pathway activation, Gpr161 is removed from the primary cilia in a Smo- and β-arrestin-dependent manner in a two-step process (Pal et al., 2016). First, β-arrestins are recruited by the signaling-competent receptor in a Grk2-dependent manner at the proximal C-terminus (Figure 2). Double knockouts of *β-arrestin1/2* or inhibition of GPCR kinase Grk2 prevents loss of Gpr161 from the primary cilia upon Shh signaling. Furthermore, Smo trafficking to the primary cilia and activation upon Shh signaling promote further β-arrestin binding and is required for Gpr161 exit from the primary cilia (Pal et al., 2016). Second, the β-arrestin-bound receptor is removed from the juxta-ciliary region by the clathrin-mediated endocytotic machinery (Pal et al., 2016). In addition to its predicted coupling to the endocytotic machinery, the distal C-tail has a conserved amphipathic helix that directly binds to type I PKA regulatory subunits (RI) (Bachmann et al., 2016) (Figure 2). As type I PKA regulatory subunit RI α localizes to cilia (Mick et al., 2015), direct coupling to PKA in this compartment might enhance Gpr161's ability to activate PKA via constitutive cAMP signaling.

A spontaneous hypomorphic allele of *Gpr161* in mice (known as *vacuolated lens* (*vl*)) with truncation of the distal C-terminus exhibits ~50% embryonic lethality showing lumbo-sacral spina bifida (Matteson et al., 2008; Wilson and Wyatt, 1986). This phenotype is extensively modifiable depending on the genetic background of the strain (Li et al., 2015), and the reasons underlying the limited penetrance spina bifida phenotypes in $Gpr161^{vl/vl}$ mice are presently unclear. The C-tail truncation in the *vl* allele is distal to the proximal C-tail β-arrestin binding site. β-arrestins bind significantly better to the *vl* truncation compared to the wild type, while binding poorly to the proximal C-tail mutant. Despite binding to β-arrestins strongly, the truncated $Gpr161^{vl}$ mutant is not removed from cilia upon Shh signaling, which is probably due to uncoupling from the endocytosis step (Pal et al., 2016). Consistent with a role of Gpr161 in negative regulation of Shh signaling, ciliary retention of Gpr161 in *β-arrestin* double knockouts, and of the β-arrestin binding site mutant (proximal C-terminus, 376–401 aa, S/T>A) receptor results in decreased Shh signaling, irrespective of Smo trafficking to the compartment. However, ciliary retention of the $Gpr161^{vl}$ truncated mutant does not result in Shh signaling defects (Pal et al., 2016). Reduced cAMP signaling resulting from β-arrestin-mediated desensitization and/or lack of coupling to RI subunits through the distal C-tail amphipathic helix in the $Gpr161^{vl}$ mutant possibly prevents suppression of

maximal Shh signaling even upon being retained in cilia (Bachmann et al., 2016; Pal et al., 2016). Thus, lack of RI coupling and local unrestricted increase in Shh signling in *vl* mice might account for the hypothesized hypomorphic nature of the allele (Li et al., 2015; Matteson et al., 2008). In addition, local perturbations in other morphogenetic pathways, including retinoic acid and canonical Wnt pathways might contribute to the caudal neural tube closure defects (Li et al., 2015). While direct PKA coupling by the amphipathic helix might function in restricting high Shh signaling, the Gpr161^{vl} mutant does not cause global Shh signaling defects in contrast to the null allele (Li et al., 2015; Mukhopadhyay et al., 2013). Thus, Gpr161 activity can persist in the absence of direct PKA coupling by the amphipathic helix. Overall, Gpr161 is a critical factor in the basal suppression machinery of Shh signaling, neural tube morphogenesis and closure.

Tulp3 and IFT-A—Factors that regulate Gpr161 trafficking to the primary cilia include the tubby-like protein 3 (Tulp3) and intra-flagellar complex A (IFT-A) (Mukhopadhyay et al., 2010) (Figure 2). Either of these knockouts result in increased Shh signaling, phenocopies *Gpr161* null mutants by exhibiting ventralization of the caudal neural tube, and exhibits NTDs including exencephaly and spina bifida (Liem et al., 2012; Ocbina et al., 2011; Qin et al., 2011; Tran et al., 2008).

Gα_s and PKA—Factors downstream of Gpr161 include the G-protein subunit Gα_s and PKA. Mutations of the gene encoding Gα_s in *Gnas*$^{-/-}$ embryos result in open neural tube and embryonic lethality by E9.5 (Regard et al., 2013). *Gnas*$^{-/-}$ embryos have upregulated *Shh* signaling and show ventralization of the neural tube (Hwang and Mukhopadhyay, 2015; Regard et al., 2013). In addition, mesoderm-specific knockout of *Gnas* (*Dermo1-Cre; Gnas*$^{fl/-}$) embryos results in exencephaly by E14.5 (~20%) (Regard et al., 2013).

PKA is a serine-threonine kinase that is activated by cAMP. PKA inhibits Shh signaling pathway (Huang et al., 2002) by processing Gli2 and Gli3 proteins to the repressor forms (Wang et al., 2000). PKA holoenzyme consists of two catalytic units, Cα and Cβ (encoded by *Prkaca* and *Prkacb*) and two regulatory subunits, type I subunits (RI α and RI β, encoded by *Prkar1a* and *Prkar1b*, respectively) and type II subunits (RII α and RII β, encoded by *Prkar2a* and *Prkar2b*) in mice (Kirschner et al., 2009; McKnight, 1991). The PKA regulatory subunit RI α localizes to cilia, while other PKA regulatory and catalytic subunits have been reported to be associated with the ciliary proteome and basal body (Barzi et al., 2010; Mick et al., 2015; Tuson et al., 2011). Although, all the regulatory isoforms are expressed in the developing neural tube (Norman et al., 2009), only *Prkar1a* knockouts exhibit open neural tube in the fore brain and mid brain regions, and are severely growth arrested to resemble E8.5 embryos before turning (Amieux et al., 2002). In mice with partial lack of catalytic subunits ($C_\alpha^{+/-}$; $C_\beta^{-/-}$; *PKA* deficient mice), 100% of embryos exhibit spina bifida and ~25% of embryos exhibit exencephaly by E12.5 (Huang et al., 2002). Complete loss of PKA ($C_\alpha^{-/-}$; $C_\beta^{-/-}$; *PKA* null mouse) results in NTDs and embryonic lethality by E9 (Tuson et al., 2011). *PKA* deficient or null embryos exhibit ventralization of the neural tube in a Gli2-dependent manner (Tuson et al., 2011).

Other factors that promote Gli3 repressor formation include Sufu and *Sufu* knockout embryos result in ventralization of the neural tube (Svard et al., 2006). Thus, lack of factors

regulating the basal repression machinery of Shh signaling pathway (Tulp3-IFT-A-Gpr161-$G\alpha_s$-PKA-Sufu) can all result in increased Shh signaling in the neural tube and NTDs (reviewed in (Hwang and Mukhopadhyay, 2015)). In addition, at least one of these factors ($G\alpha_s$) also functions in the cranial mesenchyme to regulate neural tube closure.

4.2. Protease-activated receptors and signaling through $G\alpha_{i/o/z}$ and $G\alpha_{12/13}$ subunits

Protease-activated receptors (PARs, PAR1-4) belong to the class A GPCR family that are activated by extracellular proteolysis (Arora et al., 2007). PARs are known to play a role in inflammation, thrombosis and development. PAR1, PAR3 and PAR4 are activated by thrombin (Ishihara et al., 1997; Kahn et al., 1998; Rasmussen et al., 1991; Vu et al., 1991), whereas PAR2 is activated by trypsin, mast cell tryptase and coagulation factor VIIa/Xa (Camerer et al., 2000; Mari et al., 1996; Molino et al., 1997; Nystedt et al., 1994; Riewald and Ruf, 2001). Both *Par1* and *Par2* single knockout mice die around E9 and E10, respectively, but without neural tube defects (Camerer et al., 2010; Connolly et al., 1996; Griffin et al., 2001; Szabo et al., 2014). *Par1; Par2* double knockout mice exhibit midgestational cardiovascular failure and late gestational lethality associated with generalized edema (Camerer et al., 2010). Unexpectedly, ~30% of embryos exhibit exencephaly by E11.5 and surviving mice suffer from spina bifida. Interestingly, *Par1 and Par2* mRNAs are not expressed in the neural tube, but in the surface ectoderm, just dorsal to the point of neural fold fusion. PAR family GPCRs couple with $G\alpha_{q/11}$, $G\alpha_{i/o/z}$ and $G\alpha_{12/13}$ subunits (Coughlin, 2000) and to test if which of these mediate PAR signaling in the surface ectoderm to regulate neural tube closure, conditional mouse models were generated using a predominantly ectoderm-specific cre line ($Grhl3^{Cre/+}$) (Camerer et al., 2010; Rolo et al., 2016). Embryos with conditional deletion of $G\alpha_{12/13}$ in the surface ectoderm ($Grhl3^{Cre/+}$; $G\alpha_{13}^{fl/fl}$; $G\alpha_{12}^{-/-}$) do not show NTDs, whereas inhibition of $G\alpha_{i/o/z}$ family by overexpression of pertussis toxin ($Grhl3^{Cre/+}$; $ROSA26^{PTX/PTX}$) result in exencephaly (~10%), spina bifida (~18%) and curly tail phenotypes (~40%) by E14.5 (Camerer et al., 2010). Furthermore, evaluation of candidate Par2-activating proteases in the context of neural tube closure demonstrate a local network of membrane-tethered proteases in triggering Par2 signaling in the surface ectoderm in a $G\alpha_i$-dependent manner during neural tube closure (Figure 2). Rho family GTPase function downstream of $G\alpha$ signaling pathways (Dorsam and Gutkind, 2007; Lagerstrom and Schioth, 2008), and the authors proposed that Rac1 functions downstream of Par1/2 and $G\alpha_i$ in determining neural tube closure (Camerer et al., 2010). Although Rac1 is critical in neural tube closure by promoting membrane ruffling in the apposing folds of the surface ectoderm (Rolo et al., 2016), a direct functional link between Par1/2-induced $G\alpha_i$-dependent signaling and Rac1 is currently missing. In summary, a defect in GPCR signaling in the surface ectoderm can cause failure of neural tube closure leading to exencephaly.

4.3. Lysophosphatidic acid (LPA) and Sphingosine-1-phosphate (S1P) receptors

Lysophosphatidic acid receptors (LPAR) are GPCRs that are activated by the phospholipid metabolite, lysophosphatidic acid (LPA) (Choi and Chun, 2013; Choi et al., 2010). LPA activates cell proliferation, migration and survival through LPARs (LPA1-6 receptors) in mammals (Dennis et al., 2005; Lin et al., 2010). Autotaxin (ATX, alternatively known as ENPP2) is a critical enzyme for LPA-LPAR signaling cascade. ATX is a secreted

glycoprotein and a member of the ectonucleotide pyrophosphatase/phosphodiesterase family of ectoenzymes that hydrolyzes lysophosphatidylcholine (LPC) to generate LPA (Nishimasu et al., 2011; Tokumura et al., 2002; Umezu-Goto et al., 2002). ATX is abundantly expressed in the floor plate at E9.5 (Bachner et al., 1999; Koike et al., 2011; van Meeteren et al., 2006). *Atx* knockout embryos show embryonic lethaliy by E9.5 with vascular defects in the yolk sac and failure of cranial neural tube closure (Fotopoulou et al., 2010; Koike et al., 2011; van Meeteren et al., 2006). Alterations in dorso-ventral patterning of the neural tube were not detected (Fotopoulou et al., 2010) but attenuated embryonic expression of HIF-1A was demonstrated, suggesting a novel effector pathway for ATX/LPA (Figure 2). Interestingly, ~5% of *Lpar1*$^{-/-}$ embryos exhibit exencephaly at E18.5 (Contos et al., 2000), suggesting that *Atx* deficient mice and *Lpar1*$^{-/-}$ mice partially phenocopy each other. On the other hand, *Lpar2* or *Lpar3* knockout mice are not embryonic lethal, indicating functional redundancy in LPA receptors (Choi et al., 2008).

Shingosine-1-phosphate (S1P) is a sphingolipid metabolite that regulates cell growth, survival, differentiation and motility through S1P receptors (S1PRs, S1PR1-5) that have been identified as GPCRs (Spiegel and Milstien, 2003). Sphingosine kinases (Sphk1 and Sphk2) phosphorylate sphingosine to form S1P, therefore being critical for S1P signaling pathway (Liu et al., 2000a). Notably, ~18% of *Sphk1*$^{-/-}$*; Sphk2*$^{-/-}$ embryos exhibit exencephaly by E 10.5; however, no spina bifida is observed (Mizugishi et al., 2005). Exencephaly is associated with increased number of apoptotic cells in the telencephalon, the diencephalon and the rhombencephalon and underproliferation in the telencephalon, but not in the other regions (Mizugishi et al., 2005). *S1pr1* is expressed strongly in the forebrain but weakly in the midbrain and the hindbrain at E9.5. *S1pr2* is expressed in migrating cranial neural crest cells and somites but not in the neural plate and cranial mesenchyme at E8.5 (Meng and Lee, 2009). Whole mount in situ hybridization showed that *S1pr3* is expressed in somites, the cranial mesenchymal tissues and the lateral plate mesoderm in the tail region in E8.5 and E9.5 mouse embryos, and its expression decreased by E10.5 (Meng and Lee, 2009; Ohuchi et al., 2008). *S1pr1*$^{-/-}$ mice die between E12.5–14.5 due to hemorrhages (Liu et al., 2000b), while *S1pr2*$^{-/-}$, *S1pr3*$^{-/-}$ and *S1pr5*$^{-/-}$ single knockout mice do not have any obvious abnormal growth defect at birth (Choi et al., 2008; Ishii et al., 2001; Ishii et al., 2002; Jaillard et al., 2005). *S1pr1; S1pr2; S1pr3* triple knockout mice die by E10.5–11.5 resulting from lethal hemorrhages; however, it is not clear whether they have neural tube defects (Kono et al., 2004).

4.4. CELSRs and the planar cell polarity pathway

Cadherin epidermal growth factor laminin G seven-pass G-type receptors 1–3 (CELSR1–3) belong to family B GPCRs (Harmar, 2001). CELSRs play an essential role in planar cell polarity (PCP), neuronal differentiation, and cardiovascular disease (Wang et al., 2014). CELSRs have cadherin repeat domains in the N-terminus (Wang et al., 2014). Similar to cadherin being calcium-dependent cell adhesion molecules, cell-cell adhesion plays a role in activation of CELSRs (Berger-Muller and Suzuki, 2011). Two independent N-ethyl-N-nitrosourea (ENU) mutagenesis screens identified mice with head shaking behavior and neural tube defects, named *spin cycle* (*Scy*) and *Crash* (*Crsh*), respectively, both with missense mutations in the coding region of *Celsr1* (Curtin et al., 2003; Nolan et al., 2000).

Scy$^{-/-}$ and *Crsh*$^{-/-}$ mice exhibit craniorachischisis by E8.5 and exencephaly by E12.5 (Curtin et al., 2003). *Celsr1* missense mutations have also been identified in human patients with spina bifida, craniorachischisis and caudal agenesis (Allache et al., 2012; Lei et al., 2014; Robinson et al., 2012). CELSR1 is concentrated in adherens junctions oriented toward the mediolateral axes of the neural plates, recruits Frizzled and Dishevelled resulting in localized activation of PDZ-RhoGEF and Rho kinase (ROCK), inducing actomyosin contraction along the medio-lateral axis and neural plate bending (Nishimura et al., 2012) (Figure 2). CELSR2 and CELSR3 have been proposed to activate phospholipase C and increase inositol-3-phosphate and cytosolic Ca^{2+} levels in hippocampal neurons, although the rates of activation are slower compared to typical GPCRs (Shima et al., 2007). However, *Celsr2*$^{-/-}$ embryos and *Celsr3*$^{-/-}$ embryos do not exhibit neural tube defects (Boutin et al., 2012; Tissir et al., 2010).

4.5. Other GPCR signaling proteins and their roles in neural tube development and closure

Knockout mouse models of G protein subunits other than $G\alpha_s$ and $G\alpha_i$ also show NTDs. While *Gα*$_{13}$ knockout embryos show exencephaly by E9.5 (Offermanns et al., 1997; Ruppel et al., 2005), restricted surface ectoderm-specific deletion of *Gα*$_{13}$ does not result in NTDs (Camerer et al., 2010). Deletion of *Gα*$_{9}$ and *Gα*$_{11}$ results in embryonic lethality by E11, but the role of these subunits in neural tube development and closure was not carefully characterized (Offermanns et al., 1998). *Gβ1*$^{-/-}$ embryos exhibit exencephaly but not spina bifida by E10.5 (~40% penetrance) (Okae and Iwakura, 2010). Notably, proliferation and apoptosis in the neural tube was not affected in *Gβ1*$^{-/-}$ embryos, whereas reduced level of F-actin in the dorsolateral hinge points were noted (Okae and Iwakura, 2010). *Gβ5*$^{-/-}$ mice survive to adulthood with multiple neurologic abnormalities, such as motor learning and coordination (Zhang et al., 2011), but NTDs were not observed.

Among Gγ subunit knockout mouse models, *Gng5*$^{-/-}$ embryos die by E10.5 due to failure in cardiac development (Moon et al., 2014), but their role in neural tube development is not clear. No developmental defects are reported in *Gng1*, *Gng3*, *Gng7* or *Gng13* knockout mouse models (Li et al., 2013; Lobanova et al., 2008; Schwindinger et al., 2003; Schwindinger et al., 2009; Schwindinger et al., 2004).

5. Future directions

As next-generation sequencing platforms are increasingly being used in identification of genes mutated in NTDs, it is critical to determine if the newly identified mutations contribute to NTD pathogenesis. Discovering newer GPCR-regulated pathways that regulate neural tube morphogenesis and closure allows early diagnosis to prevent neural tube defects. Furthermore, newly identified patient mutations can be tested for their role in pathogenesis of neural tube defects using knock in approaches in mice. Newer developments in CRISPR-Cas9 methodology makes it easier to generate genetically modified mice. In addition, it is important to develop *ex vivo* NTD models. Recent advances in 3D-culture and induced pluripotent stem cell-derived-organoids are promising new directions in developing neural tube models for understanding functional consequences of newly identified mutations in pathogenesis of NTDs.

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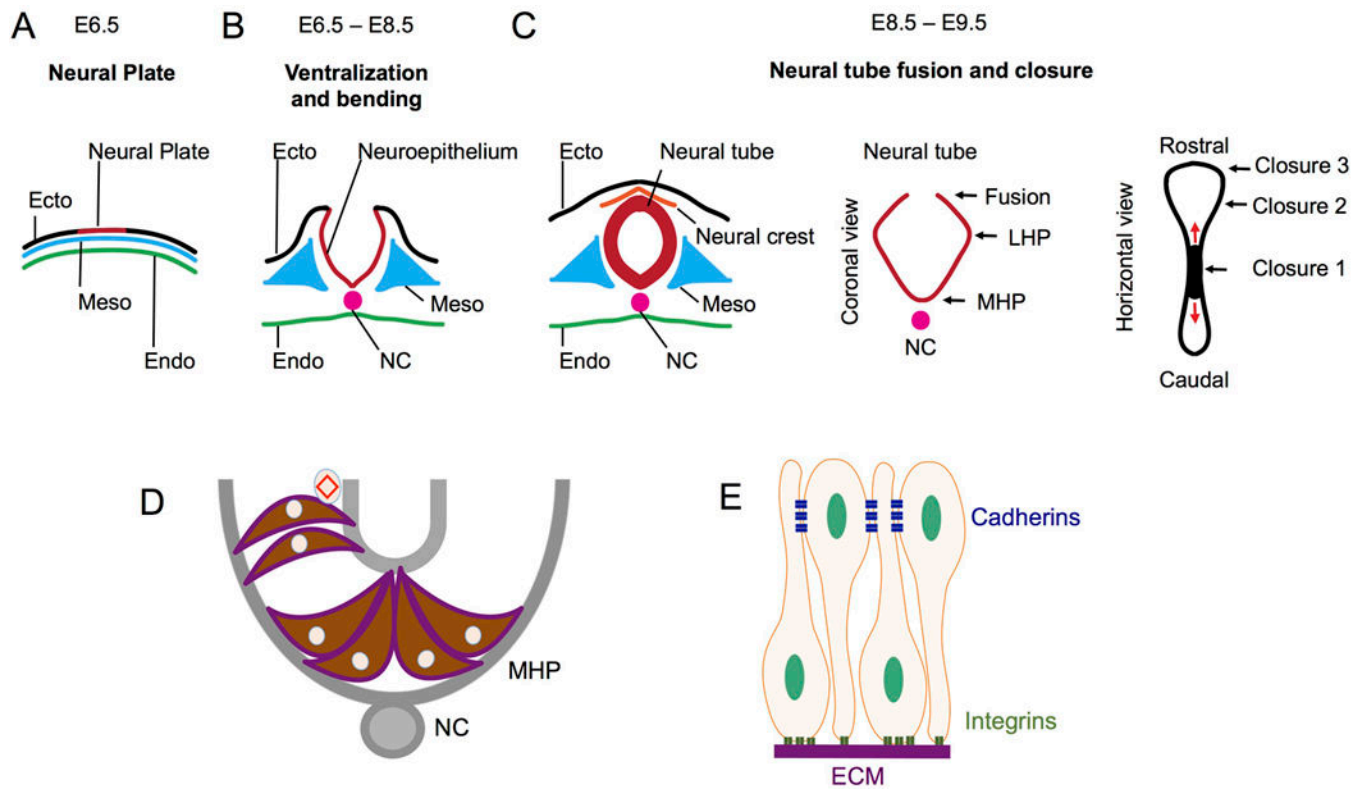

Figure 1. Summary of neural tube formation and closure

(A–C). Abbreviations: Ecto, ectoderm; Meso, mesoderm; Endo, endoderm; NC, notochord; LHP, lateral hinge point; MHP, medial hinge point. **(D)** Arrangement of neuroepithelial cells in medial hinge points. **(E)** Cell-cell and cell-matrix interactions important in the establishment of the pseudostratified neuroepithelium.

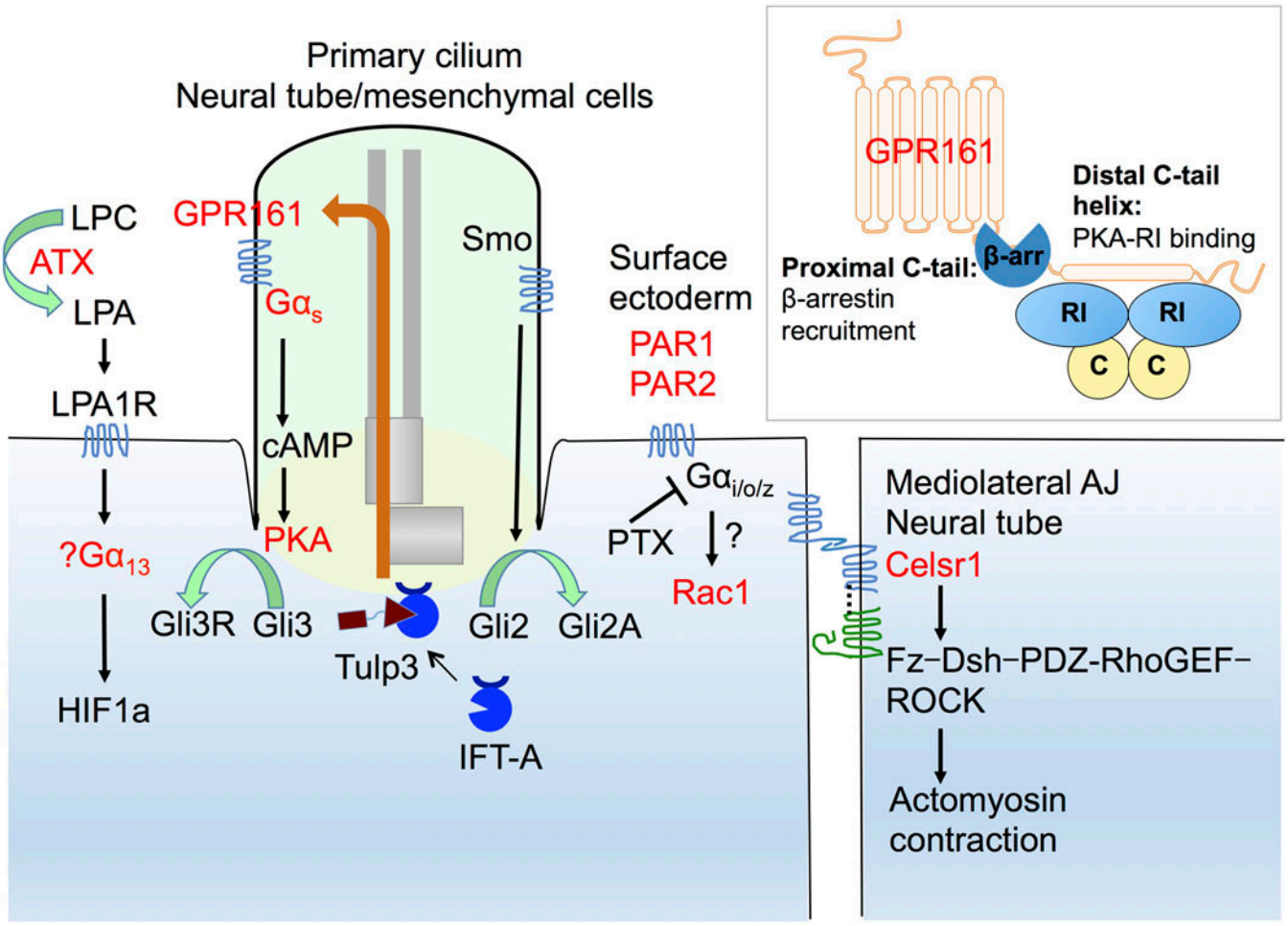

Figure 2. GPCR signaling pathways implicated in neural tube development and closure

Red color indicates genes with known mutations causing neural tube defects. Inset on right shows Gpr161 C-tail proximal and distal regions implicated in β-arrestin and PKA RI binding, respectively. Abbreviations: AJ, Adherens junction; β-Arr, β-Arrestin; C, PKA catalytic subunit; Dsh, Dishevelled; Fz, Frizzled; Gli2A, Gli2 activator; Gli3R, Gli3 repressor; IFT-A, intraflagellar complex A; RI, PKA RI subunit; ROCK, Rho Kinase; Smo, Smoothened.