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Actin Distribution in *Lamina Neuralis* During Cranial Neurulation of Wistar Rats Embryo (*Rattus rattus*)

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Abstract

The failure of morphogenetic processes during cranial neurulation is known lead into neural tube defect (NTD), such as craniorachisis and exencephaly. One of the processes is changing in lamina neuralis cells shape, which is caused by actin microfilament rearrangement within lamina neuralis cells. To examine the distribution of actin microfilament during cranial neurulation Wistar rats embryo were used. Embryos were obtain at following days of development; 8 days 18 hours, 9 days, 9 days 6 hours, 9 days 12 hours, 9 days 18 hours, and 9 days 20 hours respectively. Immunohistochemistry Avidin Biotin-peroxidase Complex (ABC) method was used to examine and identify the distribution of actin in lamina neuralis cells. Light microscopic observation shows positive reaction for actin immunoreactivity in the apical surface of bending lamina neuralis cells. In contrast, actin is not observed in non-bending lamina neuralis. Actin is not detected at 8 days 18 hours embryos. At 9 days embryos, positive reaction is observed over the entire apical surface of lamina neuralis.

Key words: Cranial neurulation, Actin, lamina neuralis, Rats embryo.

Introduction

The failure of cranial neurulation has been widely known lead into neural tube defect (NTD), such as *craniorachischis* and *exencephaly* in the knock out and mutants mouse. Much evidence has been showed that cranial neurulation is more sustainable to disturbance than spinal neurulation. Copp (2003) reported that most of teratogenic agents induced cranial neurulation rather than spinal neural tube disease.

A fundamental event of central nervous system development is cranial neurulation. Cranial neurulation is the onset of *tubulus neuralis* formation process which are occurs in cranial region. The formation of *tubulus neuralis* involves series of morphogenetic changes in the *lamina neuralis*. These morphogenetic changes are divided into two processes: intrinsic and extrinsic. The intrinsic process involves elongation and rearrangement including; alteration of the neural plate cells, cell shape changes, cell division, elevation and apposition of the neural folds and the fusion of the neural fold at dorsal midline (Davidson and Keller, 1999; Jacobson and Gordon, 1976; Schoenwolf and Alvarez, 1989; Schroeder, 1970; Van straaten, *et al*., 1993). Extrinsic processes include medial pushing on the neural folds by the adjacent epidermis, as well as inductive and mechanical contributions from the underlying mesoderm (Alvarez and schoenwolf, 1992; Brun and Garson, 1983; Jacobson and Jacobson, 1973, Van straaten, *et al*.1996).

Generally neurulation begins with the onset of morphogenetic processes. One of

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morphogenetic processes which are involved in the formation of *tubulus neuralis* is convergent extension. This extension is driven by the polarity rearrangement of cells within the tissue, which is caused by the bending of *medioventral* and *dorsolateral* parts of *lamina neuralis*. *Medioventral* bending forms *sulcus neuralis* and dorsolateral arouses *torus neuralis*. Gilbert (2000) explained that *tubulus neuralis* generates by midline fusion of *torus neuralis*. In mouse embryo bending occurs at two regions, in the midline and dorso-lateral region of *lamina neuralis*. Midline region is called Medial Hinge Point (MHP) and dorsolateral region is called Dorsolateral Hinge Point (DLHP). Gilbert (2000) and Kalthof (1996) reported, changes in cells shape involved in these bending. These cells undergo elongation, increased their height and become wedge shaped. Microfilament and microtubule are known involved in this change. These processes proceed in rostro-caudal directions of embryo.

There are specific events occur in cranial neurulation. These events are including expansion of cranial mesenchyme, *lamina neuralis* bending that depends on actin cytoskeleton, and *crista neuralis* emigration. After the midline bending the elevation of *torus neuralis* involves mesenchyme expansion. The mesenchyme will undergo proliferation which is causing the mesenchyme produce more extracellular matrix. This extracellular matrix serves as pedals for mesenchyme cell to undergo expansion. The expansions increase the extracellular space between mesenchyme cells and it results in the biconvex *torus neuralis* and increase of *torus neuralis* elevation (Copp, 2003).

Cranial neurulation is known highly dependent on actin microfilament. The role of microfilament and microtubules on chick embryo neurulation stage were investigated in vitro. Actin microfilament is involved in neuroepithelial cells surface constriction, while microtubule is involved in elongation of neuroepithelial cells (Karfunkel, 1972). The bending of cranial *lamina neuralis* is affected by *Cytochalasin D*. The *cytochalasin D* binds to (+) ends of actin microfilaments which prevent further elongation. Moris-Kay and Tuckett (1985) showed *lamina neuralis* bending failed to form after *Cytochalasin D* exposure to neurulation stage of mouse embryo in culture environment.

During embryo development actin and myosin cooperates to regulate cell shape, cytokinesis and morphogenetic changess (Jacinto and Baum, 2003). Organelle arrangement and cell shape dynamically change according to the stage of embryo development. Actin changes its arrangement and location in embryo cells during embryonic development. Examination of actin distribution in *lamina neuralis* bending during cranial neurulation is important to be conduct. Since, the evidence that cranial neurulation is highly dependent on actin microfilament rearrangement and more sustainable to disturbance than spinal neurulation. This study is examining the actin microfilament distribution pattern in bending *lamina neuralis*.

Materials and Methods

Twelve female *Wistar* rats were caged with males overnight; each cage contains 4 female and 1 male rats. Vaginal smear were examined at the next morning. Presence of vaginal plug indicated 0 day of pregnancy. The pregnant females were sacrificed using overdose of inhalation of ether. The euthanasia methods were done according to American Veterinary Medical Anesthesia Association (AVMA) (anonymous, 2007). The embryos were collected at the following day of development; 8 days 18 hours, 9 days, 9 days 6 hours, 9 days 12 hours, 9 days 18 hours, and 9 days 20 hours respectively.

Thirty six embryos were fixed in Bouin solution for three hours. They were then, dehydrated in degraded ethanol series, cleared in toluol and embedded in paraffin. Rat duodenal epthelium were used as negative control and positive control. Transverse sections (8 µm) were made on rotary *microtome* (Yamato Ry-240, Japan) and 4-5 sections adhered to gelatin subbed slides.

To examine and identify the distribution of actin in *lamina neuralis*, Immunohistochemistry *Avidin-Biotin peroxidase Complex* (ABC) was used. The Immunohistochemistry ABC method was according to Hsu and Raine (1981). The sections were deparafinized in xylol, rehydrated in degraded ethanol from 96% to 30 % and washed three times in PBS 0, 01M. Microwave antigen retrieval method was used by immersing the section with Citrate buffer pH 6.0 and incubated in domestic microwave 700 W for 10 minutes. Endogenous peroxidase activity was blocked by incubating the section with 0,3 % H_2O_2 in methanol, washed three times in PBS 0.01 M and then, incubated with nonimunized goat serum diluted 1:50 for 30 minutes at room temperature. *Monoclonal Anti-actin produced in mouse* (Sigma Cat.no: A4700) was used as primary antibody and applied for two nights in 4°C. Secondary antibody *Biotinylated anti-mouse IgG raised in goat* (Vector Laboratories Inc.) diluted 1:200 were applied in the sections for 45 minutes at room temperature, washed three times in PBS 0.01 M, and incubated with Avidin-Biotin conjugated Complex solution (ABC-Kit, Elite Vectastain® Vector laboratories, Cat No: PK-6100) for 30 minutes at room temperature. Chromogen DAB (3,3-diaminobenzidine tetrahydrochloride) containing 1% H_2O_2 applied in the sections for 25 minutes at room temperature, washed in tap water for 10 minutes and counterstained with Mayer Haematoxylin for 5 deep. The sections were then dehydrated in degraded ethanol, cleared and mounted in *canada* balm.

Result

Light microscopic examination of immunohistochemistry staining was based on color intensity appearing on the section. The color intensity scored negative (-) for negative staining or no actin was stained. It scored positive (+) for actin was weakly stained, and strong positive (++) for actin which was strong stained. The result of immunohistochemistry staining is presented in table as follows:

Table 1. Actin immunohistochemistry staining score

Sample	Immunorecativity site	Score
Positive control	Apical surface of duodenum epithelia	++
Negative control	Apical surface of duodenum epithelia	-
8 days 18 hours embryo	Apical surface of *stria primitiva*	-
9 days embryo	Apical surface of MHP	+
9 days 6 hours embryo	Apical surface of *Torus neuralis*	++
	Basal *lamina neuralis*	-
9 days 12 hours embryo	Apical surface of MHP	++
	Apical surface of *Torus neuralis*	++
	Basal of *lamina neuralis*	-
9 days18 hours embryo	Apical surface of MHP	+
	Apical surface of *Torus neuralis*	+
	Apical surface of DLHP	+
	Basal of *lamina neuralis*	-
9 days 20 hours embryo	Apical surface of MHP	+
	Apical surface of *Torus neuralis*	+
	Apical surface of DLHP	+
	Basal of *lamina neuralis*	-

Actin is known arranged along microvilli on the apical surface of duodenum epithelia. Actin immunoreactivity is observed on positive control, it is not observed on negative control (Figure 1A and B). Based on *torus neuralis* shape, *lamina neuralis* bending divided into two stage of development i.e. convex stage and concave stage. Convex stage is seen at 9 days, 9 days 6 hours, and 9 days 12 hours embryos. Concave stage is seen at 9 days 18 hours and 9 days 20 hours embryos.

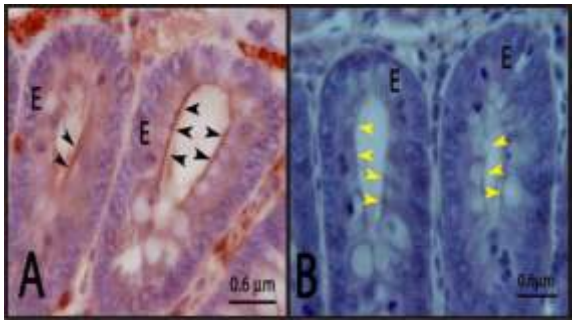

Figure 1. Transverse section of rats duodenum actin immuno-histochemical staining.
A. Positive control, rats crypts duodenum, B. Negative control,rats crypts E : Rats duodenum epithelium.(†) Shows actin immunoreactivity (†) Shows no actin imunoreactivity

Patterns of actin distribution in lamina neuralis of cranial neurulation

Actin is observed on the entire surface of *lamina neuralis* in 9 days and 9 days 6 hours embryos (Figure 3C and 3D). It is strongly stained (++) on *torus neuralis* and weakly stained (+) on MHP. Strongly stained (++) is seen on MHP, DLHP, and *torus neuralis* of 9 days 12 hours embryos (Figure 4C and 4D). In contrast, actin is weakly stained on the entire surface of MHP, DLHP and *torus neuralis* of 9 days 18 hours and 9 days 20 hours (Figure 5 and 6C, 6D and 6E). Actin is only detected on apical *lamina neuralis,* no actin is detected in basal part of lamina neuralis (non bending *lamina neuralis*). Actin is not observed on apical surface of *stria primitiva* of 8 days 18 hours embryo (Figure 2B and 2C).

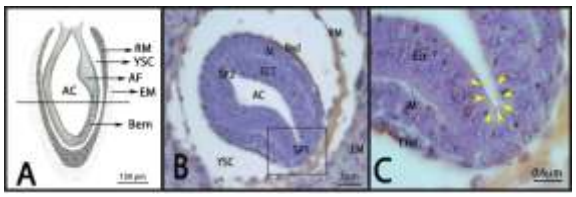

Figure 2. Eight days 18 hours rats embryo . A. a sketch of 8 days 18 hours rat embryo whole mount, ilustated the section across cranial presumptive. B. Transverse section of 8 days and 18 hours rat embryo through cranial presumtive with actin immunohistichemical staining, C. inset of figure B, shows negative stained in stria primitiva. SP1: *Stria primitiva* cranial. Ect: Ectoderm. M: Mesoderm. End: Endoderm. AC: Amnion Cavity.MHP: *Medial Hinge* Point. DLHP: *Dorsolateral Hinge Point*. LN: *Lamina neuralis*. TN :*Torus neuralis*. SN; *Sulcus neuralis*. AF: Amnion fold, YSC: Yolk Sac Cavity, RM: Reischert's membrane (†) Showed actin immunoreactivity (†) Showed no actin imunoreactivity.

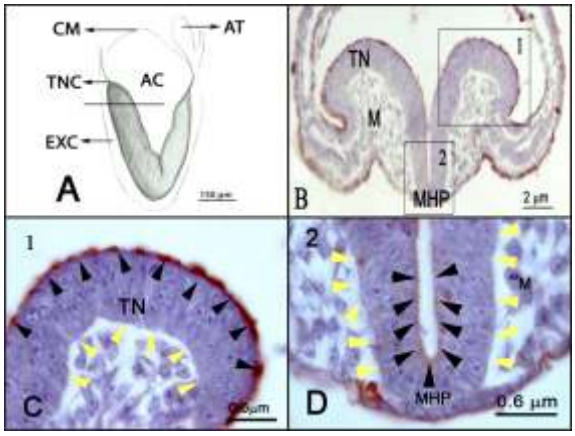

Figure 3. Nine days and 9 days 6 hours rats embryo A. a sketch of 9 days 6 hours rat embryo whole mount, ilustrated the section across cranial presumtive. B. Transverse section of days 6 hours rat embryo through cranial presumptive with actin immuno-histochemical staining. C. Inset 1 figure B Shows actin is strongly stained in *torus neuralis*. D. Inset 2 figure B shows actin is weakly stained in Medial hinge Point. (†) Shows actin immunoreactivity (†) Shows no actin imunoreactivity. AC: Amnion Cavity.MHP: Medial Hinge Point. EXC : Exoceolom cavity. TNC: Cranial Torus neuralis. CM: Chorion Membrane. AT: Alantois.

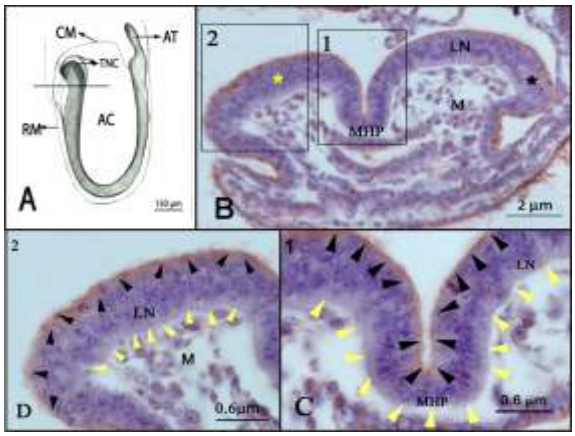

Figure 4. Nine days and 12 hours rats embryo.A. a sketch of 9 days 12 hours rat embryo whole mount , ilustrated the section across cranial presumtive

B. Transverse section of 9 days 12 hours rat embryo through cranial presumptive with actin immuno-histochemical staining. C. Inset 1figure B. shows actin is strongly stained in Medial hinge Point. D. Inset 2 figure B shows actin is strongly stained in *torus neuralis*.. (†) Shows actin immunoreactivity (†)
LN: *Lamina neuralis*. C: Cranial. BC: *Bulbus* cordis M:Mesoderm. MHP: *Medial Hinge Point*. DLHP: Dorsolateral Hinge Point. SN: *Sulcus Neuralis*. TN: *Torus neuralis*.S : Somit. AT: Alantois

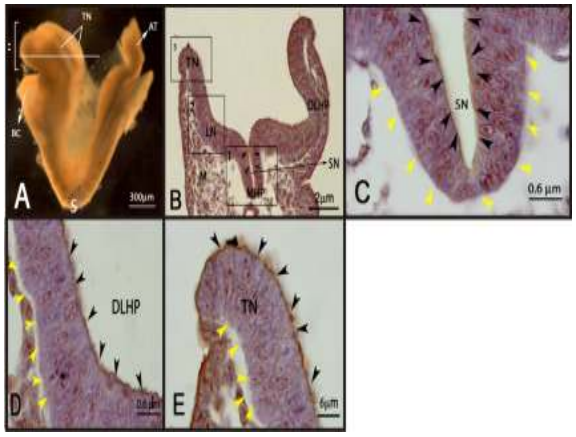

Figure 5. Nine days and 18 hours rat embryo
A. a sketch of 9 days 18 hours rat embryo whole mount, ilustrated the section across cranial presumtive. B. Transverse section of 9 days 12 hours rat embryo through cranial presumptive with actin immunohistochemical staining. C. Inset 1 figure B shows actin is weakly stained in Medial hinge Point. D. Inset 2 figure B shows actin is weakly stained in Medial hinge Point. E. Inset 3 figure B shows actin is weakly stained in Medial hinge Point.

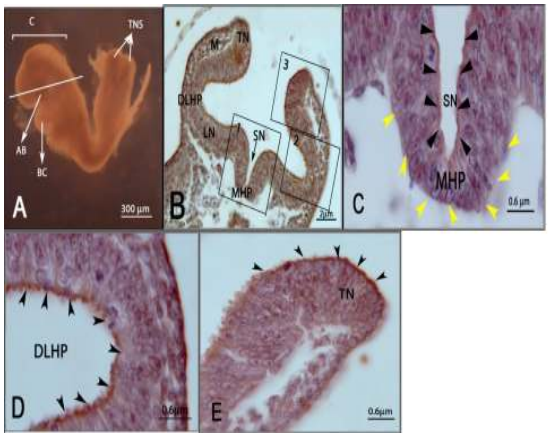

Figure 6. Nine days and 18 hours rat Embryo
A. a sketch of 9 days 18 hours rat embryo whole mount, ilustrated the section across cranial.

B. Transverse section of 9 days 20 hours rat embryo through cranial with actin immunohistochemical staining. C. Inset 1 figure B shows actin is weakly stained in Medial hinge Point. D. Inset 2 figure B shows actin is weakly stained in Medial hinge Point.E. Inset 3 figure B shows actin is weakly stained in Medial hinge Point. (†) Shows actin immunoreactivity (†) Shows no actin imunoreactivity. LN: *Lamina neuralis*. C: Cranial. BC: *Bulbus* cordis M:Mesoderm. MHP: *Medial Hinge Point*. DLHP: Dorsolateral Hinge Point. SN: *Sulcus Neuralis*. TN: *Torus neuralis*.S : Somit. AT: Alantois. AB: *Arcus Branchialis*. TNS: *Torus neuralis* spinal.

Discussion

The first immunoreactivity of actin appears on entire apical surface of *lamina neuralis* at 9 days embryo and 9 days 6 hours embryo. Actin accumulation in apical surface of *lamina neuralis* at the beginning of neurulation is to form medial hinge point (MHP). According to Morris-Kay and Tuckett (1985) after MHP has developed, contraction of apical microfilament bundles play an essential role in maintaining the elevation of the *torus neuralis*,. *Torus neuralis* will then flatten and bend on *dorsolateral* region to form *dorsolateral* hinge point (DLHP). For this bending, most of the apical surface of *torus neuralis* cells must constrict to form wedge shape cells. Therefore, compared to *torus neuralis* region, MHP is weakly stained. In latter development, t*orus neuralis* will invaginate to dorsolateral side to form DLHP. To prepare the formation of DLHP; its formation needs abundance of wedge shape cells in either MHP or *torus neuralis*. For this reason, actin was strongly stained in both MHP and *torus neuralis* at 9 days and 9 days 12 hours embryo.

Embryo development is a dynamic process; organelle arrangement and cell shape dynamically change according to the stages of embryo development. In developing embryo actin changes its arrangement and location on embryo cells during embryo development. It can be polymerized and de polymerized

depends on the developmental needs. In the present study, in 9 days 18 hours and 9 days 20 hours embryos; actin is weakly stained in MHP, DLHP and *torus neuralis*. This assumed, the accumulation of actin on apical surface is no longer needed neither for maintaining the elevation of *torus neuralis* nor to form bending. Actin serves only to maintain the MHP, DLHP and *torus neuralis* structure. In addition, less actin is polymerized on apical surface of MHP, DLHP and *torus neuralis*. This condition is in contrast to the accumulation of actin in 9 days 12 hours embryos, at those stage actin is strongly needed to prepare the formation of DLHP. Therefore, actin strongly stained on entire surface MHP and *torus neuralis* cells of 9 days 12 hours embryos.

Actin is showed weakly stained on apical part *torus neuralis* of 9 days 18 hours and 9 days 20 hours embryos, prepared for *torus neuralis* closure. Actin not only involved in *lamina neuralis* bending but also in *torus neuralis* fusion. The formation of *lamillipodial* on apical surface or *torus neuralis* is involved in the closure. *Lamillipodial* is cytoplasm protrusion formed by actin polymerization. As *torus neuralis* moved into *mediodorsal*, cellular protrusions extend from both ends of *torus neuralis* in the *mediodorsal* and fuse. Carbohydrate envelope on *torus neuralis* cells membrane is expected involved in *torus neuralis* fusion. Moran and Rice (1975) reported that the formation of lamillipodial is accompanied by carbohydrate axtrusion on cell membrane. The extrusions probably allow cell–cell recognition and provide an initial adhesion before more permanent cell contacts are established. The absence of actin immunoreactivity is showed in 8 days 18 hours embryo. This result may indicate that actin has not been polimerized to apical surface of *lamina neuralis*. This finding is supported by Takeuichi and Takeuichi (1980) an electron microscopy study of *lamina neuralis* ultra structure. Actin was not observed in *stria primitiva* stage embryo.

Eph tyrosine kinase assumed involved in *torus neuralis* fusion. Eph tyrosine kinase is Ephrin ligand on cell membrane surface for cell-cell interaction. Holemberg *et. al.* (2000) reported embryos with a null mutation of ephrin-A5, which is a ligand of glycosylphosphatidylinositol (GPI) cell surface, produce a neural tube defect an *exencephaly* embryo. Inactivation of the EphA7 receptor also produces exencephaly. Both ephrin-A5 and EphA7 are expressed in the cranial neuroepithelium before and during *tubulus neuralis* closure.

Actin is detected only on apical surface of bending *lamina neuralis*, it is not detected on non bending *lamina neuralis* (basal part of *lamina neuralis*). This condition shows actin is available in depolymerize state. Becker (2000) reported; actin (Filamentous actin) which is G-actin (Globular actin) polymer serves as dynamics structure. Depending on how the cell is formed, the G-actin will polymerize into F-actin. In depolymerize state actin is available as G-actin monomer and dispersed on the entire cell cytoplasm. *Monoclonal anti actin produced in mouse* antibody (Sigma Cat. No. 4700) which is used in this study, only recognize the epitope on C-terminal of F-actin, therefore it cannot detect the epitope on G-actin.

The failure of convergent extension in *tubulus neuralis* bending may lead into neural tube defects. Convergent extension which is involved in *tubulus neuralis* bending is driven by the polarity rearrangement of cells within the tissue. *Wnt* protein has been known responsible for convergent extension. From genetics examination is resulted *Dishevelled* gene that encoded for cytoplasmic protein, the gene is known as an essential component either *canonical Wnt* or *noncanonical Wnt* signaling, the PCP signaling cascades and also signal via *Wnt*/Ca^{2+} pathway (Gilbert, 2006). The

protein involves in biological processes such as cell polarity and cell fate specification, and even social behavior. In the *canonical Wnt* pathway, *Dsh* is important in transmitting a signal initiated from *Wnt* binding to its receptor *Frizzled* and co-receptor *Arrow/Lrp5/6* on the cell surface. As a consequence, the cytoplasmic protein Armadillo / â-catenin becomes stabilized and is transported into the nucleus to activate transcription (He et al., 2004; Wang and Wynshaw-Boris, 2004).

Xenopus Dishevelled (Xdsh) has been reported control the cell polarity via vertebrate cognate of planar cell polarity (PCP) cascade (Tada and Smith, 2000; Wallingford *et al*., 2000). The disruption of *Xdsh* signaling results in a failure of both neural convergent extension and neural tube closure (Wallingford and Harland, 2001). Furthermore, Wallingford (2002) reported that *disheveled* is needed for convergent extension and neural tube closure in *Xenopus* embryo. The *disevelled* mouse gene (*Dvl*2 and *Dvl*3) have been identified and reported required for lengthening and narrowing of neural plate and for morphogenetic changes convergent extension. In addition, *Looptail*, *Dvl1*$^{-}$, *Dvl2*$^{-}$, *Fzd3*$^{-}$, *Fzd6*$^{-}$, *Crsh* and *Scy* mouse mutant shows severe neural tube defects in which the entire neural tube from mid-brain to tail fails to close. They show a congenital defect termed *craniorachischisis* in human (Wang, *et al*., 2006).

The arrangement of cytoskeleton in the cell has been known controlled by *noncanonical* signaling pathway. In such signaling pathway, *Wnt* protein activates *Frizzled* in the way that causes *Frizzled* to activate *disheveled* protein. It activates *Rho A* and *Rac* which coordinate cytoskeleton and gene expression. Rho A will activate ROCK protein to proceed transcription for cytoskeleton arrangement. The Rac protein will activate JNK protein and precede transcription in similar way as Rho A (Gilbert, 2006). For studying of actin arrangement in neurulation, further examination of Wnt signaling need to be conducted. There are two questions need to be addressed, first which process of *lamina neuralis* bending is require *Dvl* signaling. Second, whether *Dvl* signalling is through *Rac* or *Rho A* protein that involves in cytoskeletal arrangement during cranial neurulation.

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