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Tae Youn Kim March 15, 2017

Understanding the Mechanism of Neural Cell Fate Recovery in *Inpp5e*^{M2} Mice Mutant

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Abstract

Understanding the Mechanism of Neural Cell Fate Recovery in *Inpp5e*^{M2} Mice Mutant
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A crucial question in the study of vertebrate development is defining the cellular and molecular mechanisms of how embryonic cell fates are reproducibly patterned. For example, vertebrate hedgehog signaling is critical regulator of embryonic cell fate and patterning. Sonic hedgehog (Shh), one of three vertebrate Hh ligands, controls an essential signaling pathway that regulates ventral neural tube patterning. Previous research indicates that a phosphoinositide 5-phosphatase, *Inpp5e,* regulates Shh signaling. By using forward genetic screening, we isolated a novel allele of *Inpp5e* called *Inpp5e*^{M2}. Homozygous *Inpp5e*^{M2} mice showed expanded vental neural tube cell fates during embryonic development (embryonic day 9.5 and 10.5). Surprisinly, using immunofluorescence, I found the abnormal patterning of *Inpp5e*^{M2} at E10.5 partly recovered over time by E12.5. Previous work from the lab studied the conditional allele of *Arl13b*, which associates with Inpp5e, and showed *Arl13b* mutants can correct neural patterning over time. Furthermore, this "recovery" depends on Gli3, a negative effector of Shh signaling pathway. With accumulating evidence of *Inpp5e* being an effector of *Arl13b,* I hypothesize *Inpp5e*^{M2} recovery is also dependent on Gli3 activity. To genetically test this, I aimed to generate double mutant of *Inpp5e*^{M2} ; *Gli3*^{flox}. Using the immuno-fluorescent antibody staining at E12.5, we tried to observe whether the double mutant show mispatterning neural tube. Taken together, if the double mutant result shows mispatterning of neural tube, then the recovery of patterning in *Inpp5e*^{M2} is due to *Gli3*.

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Introduction

A central question in the study of vertebrate development to define the cellular and molecular mechanisms of how embryonic cell fates are reproducibly patterned. For example, neurons in the embryonic spinal cord are correctly positioned so that neural circuits can subsequently be formed properly. As many scientific studies indicate, the determination of cell fates in the spinal cord occurs during embryonic development in a process called neural patterning. Neural patterning occurs along the dorso-ventral axis (D-V) due to activity gradients created by signaling pathways. Here, I propose to study a mouse mutant that initiates neural tube patterning incorrectly but self-corrects over time. My goal is to explore the mechanism underlying this correction of neural patterning.

During growth of the embryo and development of the neural tube, patterning is visualized by specific protein expression, in specific domains. Following the specification of the germ layers during early embryonic development, the process of neurulation initiates neural tube formation (Copp et al., 2003; Greene and Copp, 2009). The neural ectoderm forms a plate that bends in. Then two neural plate borders meet up forming the neural tube and neural crest cell dorsal to the neural tube. The neural tube is then patterned or cells are specified along with the D-V axis. Through the action of two opposing signaling pathways: Sonic Hedgehog (Shh) and Bone Morphogen Protein (BMP), the cell fates are established. During this time, any disruption of protein expression or signaling can lead to defects in the patterning of the neural tube and spinal cord function.

From earlier studies, it is clear that Shh signaling is a crucial cue for the development of spinal cord, limbs, cerebellum and craniofacial structures. One important role of Shh signaling is that it induces the ventral cell fates specification in the neural tube. This Shh signaling is

mediated by activators and repressors form. As Figure 3 indicates, Shh activity is primarily mediated by the activator for ventral cell fate and requires the repressor gradient to sustain more dorsal fates. In the ventral neural tube, a combination of the amount and duration of Shh signaling specifies six neural progenitor cell fates (Briscoe and Ericson, 2001; Echelard et al., 1993; Ericson et al., 1997; Jessell, 2000; Lee and Jessell, 1999). At the ventral midline of the neural tube, the floor plate, Shh is at its highest activity level. The high levels of Shh in the floor plate induce FoxA2 expression. The domain that lies adjacent to the floor plate, expresses Nkx2.2. Motor neuron precursors express Olig2 and HB9. Nkx6.1 is expressed in multiple domains in the ventral neural tube. Figure 1 immunofluorescent images, shows the patterning or cell fate of each neural progenitor protein.

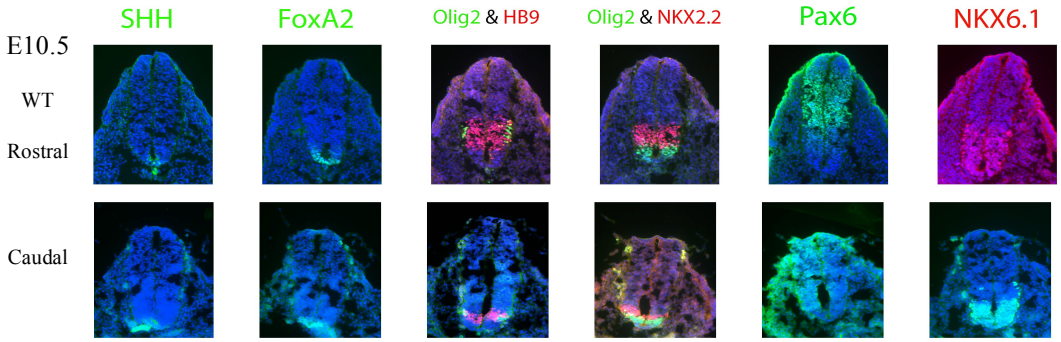

Figure 1. Rostral (top) and caudal (bottom) view of neural tube patterning of wild-type embryo of day 9.5. The protein expression is shown in either green or red as indicated in the name above. Shh, FoxA2, Olig2, HB9, Nkx2.2, Pax 6 and Nkx 6.1 patterning is indicated as above.

Shh signaling pathway is an unusual pathway in that presence or absence of protein ligands creates activator and repressor transcriptional mediators. In vertebrates, primary cilia are required for Shh signal transduction. In the absence of Shh, Ptch1, a Shh receptor, is localized to the cilium, whereas Smoothened (Smo), a downstream effector, is repressed(Corbit et al., 2005; Marigo and Tabin, 1996; Rohatgi et al., 2007). Gli, a downstream transcription factor, is cleaved to be a repressor form (GliR), inhibiting the target genes. However, when Shh ligand is present,

Smo becomes enriched and activated in cilia, and Gli proteins are cleaved to an activated form (GliA) that induces transcription of target genes. The delicate balance of GliR and GliA ultimately dictates the output of the pathway, and both under- and over-activation of Shh signaling are detrimental (Bay SN and Caspary T, 2012).

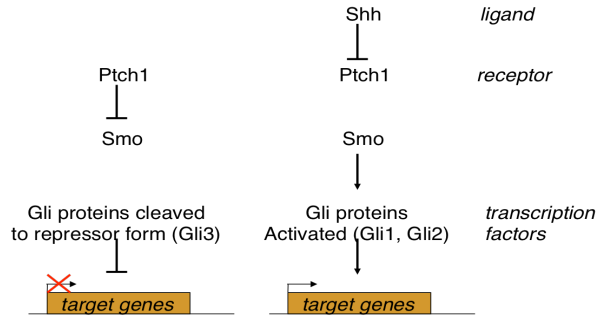

Figure 2. Presence or absence of protein ligands creates activator and repressor transcriptional mediators in Shh pathway.

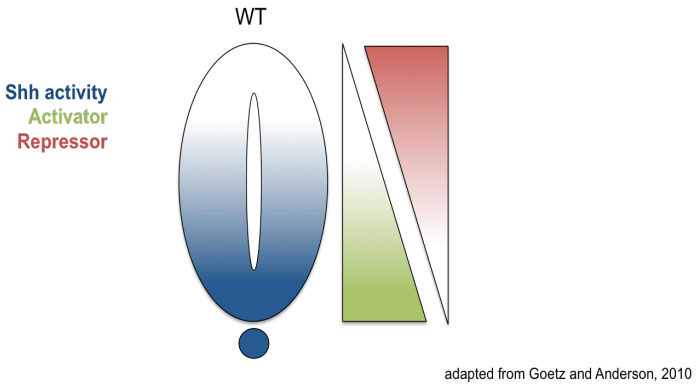

Figure 3. Shh activity is primarily mediated by the activator for ventral cell fate and requires the repressor gradient to sustain more dorsal fates.

The Caspary lab performs forward genetic screens to identify novel genes that control neural patterning. In this approach, they have used the chemical mutagen N-ethylnitrosourea (ENU) to randomly induce the mutations in mice and then screen animals phenotypically for the identification of the recessive alleles. In the course of their screens, they isolated *Inpp5e*^{M2} mouse mutants. My research focuses on *Inpp5e*^{M2}.

Inpp5e, a ciliary phosphoinositide 5-phosphatase, is an important ciliary protein for the composition of the ciliary structure. From previous work, *Inpp5e* is concentrated within the primary cilium (Jacoby et al., 2009). At the ciliary membrane, there is a particular phosphoinositide, PI(4)P, whereas a different phosphoinositide, $PI(4,5)P_2$, is restricted to the membrane of the ciliary base (Garcia-Gonzalo et al., 2015). The loss of *Inpp5e* function in mice results in malformation and malfunction of primary cilia. Mutations have also been identified in the human *Inpp5e* gene that are responsible for ciliopathies such as Joubert or MORM syndrome (Hildebrandt et al., 2011).

In addition, one line carrying a novel allele of *Inpp5e*, called *M2*, is a point mutation (D511G) of aspartic acid to glycine change at residue 511, an evolutionarily conserved residue. This mutation is clustered within the enzymatically-active phosphatse domain suggesting that mutation can alter enzymatic activity (Bielas, S.L et al., 2009). Furthermore, *Inpp5e*-null mice have been characterized and share several aspects of the *M2* phenotype, the most obvious being the exencephaly we see in both the *M2* and *Inpp5e*-null mice (Jacoby et al., 2009).

Moreover, studies reveal that *Inpp5e* is critical for the Shh signaling. *Inpp5e* is the important factor that keeps the $PI(4,5)P_2$ levels low. *Inpp5e* knockout mice in primary cilia showed disappearance of PI(4)P due to accumulation of Tulp-3 and intraflagellar transport A (IFT-A) that binds to $PI(4,5)P_2$, and which, in turn, recruits Gpr161 (Garcia-Gonzalo et al., 2015), a negative regulator of Shh signaling (Mukhopadhyah et al., 2013) . Taken together, *Inpp5e* mutation decreases the Shh response.

However, unlike previous studies where decrease of Shh response was observed in Mouse Embryo Fibroblasts (MEFs), in vivo in the neural cells of $Inpp5e^{M2}$, we observed an expansion of Shh-dependent cell fates indicating an increase in Shh response. Furthermore,

strikingly, we observed the recovery of this neural mispatterning at E12.5 in *Inpp5e*^{M2}. Through an unknown mechanism, disrupted neural tube patterning at E10.5 recovered by E12.5.

An important clue for what might control the recovery of neural tube patterning in *Inpp5e*^{M2}embryos at E12.5 comes from previous work in the Caspary lab on the *Arl13b* mouse mutant. Of note, the *Arl13b* and *Inpp5e* are both localized to cilia in same protein complex and accumulating evidence support that *Inpp5e* as an effector of *Arl13b*. This means that *Inpp5e* acts downstream of the *Arl13b*.

The Caspary lab previously observed neural patterning recovered in *Arl13b* conditional mice similar to the recovery in *Inpp5e*^{M2} embryos. When *Arl13b* was deleted at E9.25 or E9.5 (*Arl13b*$^{E9.25}$ and *Arl13b*$^{E9.5}$) embryos, abnormal patterning in the neural tube recovered by E12.5. They interpreted this to show that if the cells are initially exposed to a normal Shh gradient, then recovery is possible. In addition, they wanted to know whether GliR, *Gli3* being the primary repressor in the neural tube, gradient plays role in this phenotypic recovery. By deleting *Gli3* simultaneously with *Arl13b*, they tested whether recovery is depended on *Gli3*. In *Arl13b ; Gli3*$^{E9.25}$, they initially found the neural tube was mispatterned as in *Arl13b*$^{E9.25}$ and *Arl13b*$^{E9.5}$. By E12.5, they found the mispatterning persisted in *Arl13b ; Gli3*$^{E9.25}$ indicating that the recovery is dependent on *Gli3*.

Based on the similarity of *Arl13b* conditional and *Inpp5e*^{M2} phenotypes recovering by E12.5, I hypothesize that *Inpp5e*^{M2} recovery is also Gli3 dependent. I will test this genetically with a double mutant of *Inpp5e*^{M2} ; *Gli3*$^{E9.5}$. The *Gli3*$^{E9.5}$ allele will provide me temporal control of Gli3 deletion. If I find that mispatterning exists at E12.5, then the recovery is *Gli3*-

dependent. If I find the normal patterning at E12.5, I would then need to conclude that it is not *Gli3*-dependent and consider other possible factors or models.

Materials and Methods

Mouse Information

Dr. Tamara Caspary's lab performs forward genetic screens to identify novel genes that control neural patterning. Horner and colleagues performed a recessive ENU mutagenesis, where they have isolated 17 novel mutations, which have clear defects in neural development. Mutagenized C57BL/6J mice were crossed to FVB/NJ. G2 females were backcrossed with G1 males to generate G3 embryos, which were observed at E10.5 for abnormal development of the nervous system. Through a method of genome scan, single nucleotide polymorphism (SNP) or simple sequence length polymorphism (SSLP) markers, M2 was isolated and it was found to be linked to chromosome 2.

PCR (Genotyping)

3 different PCR protocols were used to genotype M_2, *Tom-Cre* and *Gli3*. DNA samples were mixed into dH_2O diluted at 1:5. Diluted DNA samples were then mixed with PCR master mix, which includes following: dH_2O, primers, PCR mix and Choice Taq. The following primers were used (5'–3'): M_2 (ATCAGAGCAGATGAGGGGAGGCCAG and CTGCAACACAGGAGAGTCAGGGAGG); *Tom-Cre* (TGACCCGGCAAAACAGGTAGTTA and TTCCCGCAGAACCTGAAGATGTT); *Gli3* (CTGGATGAACCAAGCTTTCCATC, CTGCTCAGTGCTCTGGGCTCC, and CTTCGTATAGCATACATTATACG [deletion primer]).

Tamoxifen Injection

Tamoxifen was dissolved to 10mg/mL in 100% ethanol. For *Inpp5e* at E10.5, 0.1mg of tamoxifen per 1 g body weight was dissolved in 300 µl of corn oil using the vacuum centrifuge for 20 minutes. Pregnant females were injected once at noon on the indicated day.

Mouse Embryo Dissection

Tamoxifen injected pregnant females with embryos sacrificed and E12.5 embryos harvested at around 10 am. Once the respiratory arrest is confirmed, of the female the mouse was laid on the absorbent pad and soaked with ethanol on the ventral side of the abdominal area for prevention of contamination due to mice hair. A small horizontal incision right above the vaginal was made and cut deep until the guts were exposed. Then lateral incision along the small horizontal incision was made to reveal the abdomen cavity. Uterine horn was removed and dissected embryos were placed in ice-cold PBS. Each embryo was separated by its implantation sites. With micro dissecting watchmaker's forceps, embryo layer was peeled off until embryo was exposed. Yolk sacs were torn off for the genotyping. With wide opening plastic pipette, mouse embryos were transferred to the prepared plate with PBS carefully.

Mouse Embryo Embedding and Sectioning

Embryos were fixed with 4% of paraformaldehyde (PFA) in phosphate buffer saline (PBS) for 1.5 hours at 4 °C. Fix was removed by washing embryos 2 x 10 minutes in PBS at room temperature. Embryos then were placed in 30% sucrose overnight at 4 °C fridge. Embryos were washed three times with OCT to remove excess sucrose. Then embryos, embedded in the

small cube with OCT in axial position, froze on dry ice and placed at -20 °C for sectioning on the cryostat in 10-micron sections.

Phenotypic Analysis – Immuno-fluorescent Staining

Each slide was washed with PBS for 10 minutes, then they were blocked with the antibody wash solution (1% Heat-inactivated goat or sheep serum, 0.1% Triton X-100, and PBS) for an hour. Primary antibodies were prepared by diluting to antibody wash solution as following: mouse anti-Shh, mouse anti-FoxA2, mouse anti- HB9, mouse anti-Nkx 6.1 were diluted at 1:10 while mouse anti-Nkx2.2 at 1:5. In addition, rabbit anti-Olig2 was diluted at 1:300. 250 µl of diluted primary antibody solution was pipetted on each slide. Then each slide was covered with parafilm to prevent loss of solution and incubated in dark flat humidifeid chamber overnight at 4 °C.

After the incubation period, the secondary antibody dilutions were prepared. First, the slides were washed 3 times for 20 minutes with antibody wash solution at room temperature. The secondary antibody solutions were diluted with wash solutions with followings: goat-anti-mouse Alexafluor 488 (Green) or donkey anti-rabbit Alexafluor 594 (Red), each with 1:200. Also, Hoechst stain solution was also diluted at 1:300 with antibody wash solution. 250 µl of secondary solution was applied to each slide in dark flat humidified chamber and protected from the light for about 2 hours at room temperature. Slides were then washed 2x30 minutes with antibody wash solution at room temperature. Then slides were mounted with ProLong Antifade reagent and examined under the microscope of Leica DM6000B. Q-capture and Simple PCI software were used to capture images. Adobe Photoshop was used to crop and adjust color, brightness, and contrast of images.

Quantitative Analysis

With the software called ImageJ, and three different color channels of staining, cells were counted. For the each image, the ratio of the cell expressing the progenitor marker or motor neuron marker to the whole neural tube cell was created.

Results

Disrupted neural patterning of *Inpp5e*^{M2} phenotype at E10.5

The Shh signaling disruption due to *Inpp5e* mutation was clearly observed in the neural patterning of mice at E10.5. As figure 4 indicates, the caudal neural tube image of the mouse embryo at E10.5, the progenitor markers and the motor neuron maker both expaneded dorsally. Progenitor markers of Olig2, Nkx2.2 and Nkx 6.1, as well as the motor neuron marker of HB9 in *Inpp5e*$^{M2/+}$ (control), were restricted ventrally. Nkx2.2 was expressed at p3 cells, Olig2 and HB9 in the pMN cells, and Nkx6.1 in the ventral cell domain up until the p2 cells.

However, in the *Inpp5e*^{M2}, expansion and mispatterning of the progenitor markers as well as the motor neuron marker were observed. In the image, Olig2, Nkx2.2, HB9 and Nkx6.1, they all showed expansion of vetral cell fates. This indicates disruption of Shh signaling.

An important fact to note from this image is not only the expansion of the progenitor makers but also the shape of the neural tube. Compared to the *Inpp5e*$^{M2/+}$ neural tube, *Inpp5e*^{M2} neural tube looks smaller, and also hollow.

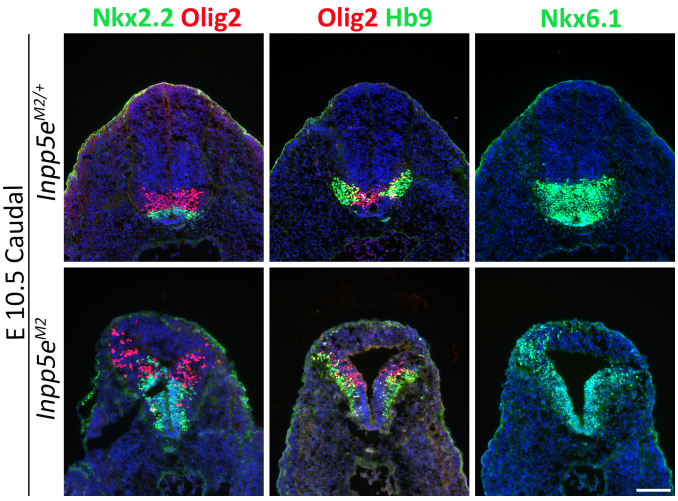

Figure 4. Neural patterning of wild-type and *Inpp5e*^{M2} caudal view at E10.5. WT indicates normal patterning of the ventral cell fates. Neural development occurs in a rostral and caudal manner, thus rostral being further developed than caudal. For *M2*, progenitor marker of HB9, Nkx2.2, Nkx6.1 are all expanded dorsally.

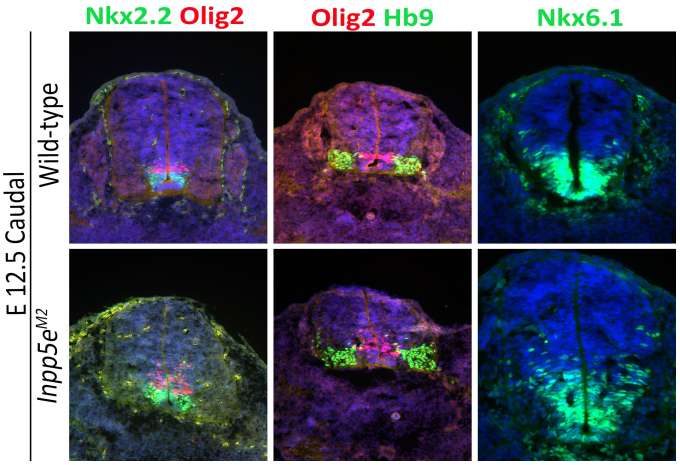

Figure 5. Neural patterning of wild-type and *Inpp5e*^{M2} caudal view at E12.5. WT indicates normal patterning of the ventral cell fates. For *M2,* unlike what we saw on at E10.5, recovery of the neural patterning is observed for HB9, Nkx2.2, Nkx6.1, and Olig2. No mispatterning and no dorsalization of neural patterning observed.

Recovery of neural patterning of *Inpp5e*^{M2} phenotype at E12.5

As figure 5 illustrates, we saw the constitutive model of *Inpp5e*^{M2} neural patterning partially recovering by E12.5. This self-recovery of the neural patterning was very surprising. In figure 5 of *Inpp5e*^{M2} stained with Nkx2.2; Olig2 and HB9; Olig2, neural patterning looks similar to that of the wild type. However, we still observed some dorsal expansion of Olig2

markers in the both image, indicating that the recovery is not perfect. In addition, unlike Nkx2.2, which was restricted at the ventral midline of the neural tube, the HB9 showed mildly expanded expression. Nonetheless, compared to the figure 4 of *Inpp5e*^{M2}, it is clear the expression of both progenitor marker and motor neuron maker are both restricted at the ventral neural tube cell domains.

In addition to the self-correction of the expression of the neural tube, the shape of the neural tube also recovered. Unlike the figure 4 of *Inpp5e*^{M2}, the figure 5 of *Inpp5e*^{M2} at E12.5 shows the normal shape of the neural tube.

This self-recovery of the neural patterning was especially surprising because compared to the recovery seen in the Arl13b conditional mice, where the recovery depended on the timing of *Arl13b* deletion, the *Inpp5e*^{M2} allele is constitutive. The findings indicating that *Arl13b* is required for *Inpp5e* cilia localization (Humbert et al., 2012, Thomas et al., 2014), hinted us that this recovery might also due to the Gli-repressor gradient, based on the *Arl13b* phenotype being *Gli3* –dependent.

To test whether this constitutive *Inpp5e*^{M2} neural tube recovery is due to GliR, we conditionally deleted the major repressor *Gli3* at E9.5, in *Inpp5e*^{M2} mutants. We first obtained the image of *Inpp5e*^{M2} ; *Gli3*$^{+/-}$. As the image indicates, the both progenitor markers and motor neuron markers partial recovery was observed.

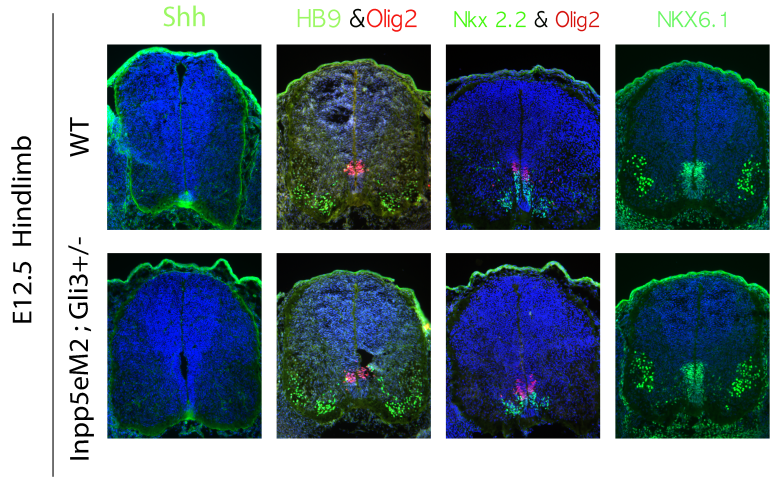

Figure 6. Neural patterning of wild-type and *Inpp5e*^{M2} ; Gli3$^{+/-}$ caudal view at E12.5. WT indicates normal patterning of the ventral cell fates. For the double heterozygous mutation, unlike what we saw on at E10.5 of figure 4, recovery of the neural patterning is observed for Shh, HB9, Nkx2.2, Nkx6.1 and Olig2. No mispatterning and no dorsalization of neural patterning observed.

As indicated in figure 6, the single deletion, heterozygous, Gli3$^{+/-}$ and *Inpp5e*^{M2} mutation at E12.5 shows the recovery of the neural patterning. Shh was expressed in the floor plate. Motor neuron marker of Olig2 and HB9 were expressed in pMN domain while Nkx 6.1 was expressed at p_2 domain and Nkx2.2 at p_3.

Discussion

The fact that dirupted neural tube patterning somehow self-recovered overtime as we see in constitutive *Inpp5e*^{M2} is very striking. Motor neuron markers such as HB9, which was outside the domain of pMN at E10.5 corrected its expression by E12.5. Unfortunately, despite my best efforts I did not obtain a double mutant *Inpp5e*^{M2} ; *Gli3*$^{E9.5}$embryo. Thus whether recovery of the constitutive neural patterning phenotype of *Inpp5e*^{M2} is *Gli3*-dependent remains unknown.

For the future work, it would be more efficient if we have *Gli3* null allele at the first place. For our research, we have crossed *Inpp5e*$^{M2/+}$; *Gli3*$^{flox/+}$; *Cre*$^{+}$ x *Inpp5e*$^{M2/+}$; *Gli3*$^{flox/flox}$.

Our chance of getting the target embryo was 1 out of 16. In addition to the probability, injecting Tamoxifen into the pregnant mother narrowed our chance of getting the target embryo due to its toxicity and complex steps requiring the Cre^{ER}.

To test this double mutant directly, we can set up cross of $Inpp5e^{M2/+}$; $Gli3^{flox/flox}$; Cre^{+} x $Inpp5e^{M2/+}$; $Gli3^{flox/flox}$. This would double the probability of obtaining the embryo we want. Another option is to cross $Inpp5e^{M2}$; $Gli3^{flox/+}$ x $Inpp5e^{M2}$; $Gli3^{flox/+}$. The probability of obtaining the target embryo remains same but has the advantage of skipping Tamoxifen injection. If in the further research that $Inpp5e^{M2}$; $Gli3^{E9.5}$ shows the mispatterned neural tube phenotype at E12.5, this means that recovery is *Gli3*-dependent. However, if the double mutant shows recovery without any mispatterning, this would mean, it is not *Gli3* dependent and would have to further extrapolate what mechanism this recovery is occurring.

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