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Effects of an Impaired Sonic Hedgehog Signaling Pathway and a Nonfunctional Gli3 Protein on GnRH-1 Neuronal Migration in Gli3Xt/Xt mutants

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mutants

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Abstract

Gonadotropin releasing hormone (GnRH) is the master regulatory hormone for sexual development. During embryonic development, gonadotropin releasing hormone-1 neurons (GnRH-1ns) form in the olfactory pit and migrate, along axonal Peripherin positive fibers, from the nasal area to the pre-optic area of the basal forebrain. Upon migration into the brain, GnRH-1ns release GnRH. Defective migration of GnRH-1ns can result in hypogonadotropic hypogonadism (HH), a condition that results in lack of sexual development and infertility. When HH appears associated with reduced or absent sense of smell, it is clinically defined as Kallmann Syndrome (KS) (Paolo E Forni & Wray, 2015). The neurons that connect the nasal area to the basal forebrain and the molecular mechanisms that control GnRH-1 neuronal migration are still largely unknown. Sonic hedgehog (Shh) plays important roles in modulating cell motility and reactivity to chemorepellants. Gli3 is a transcriptional effector; it can act as either a transcriptional activator or repressor, mediated by the Shh signaling pathway. We found that Patched-2, a receptor for Shh, is expressed along the GnRH-1 migratory pathway. To understand if Shh and Gli3 play a role in GnRH-1 development, we analyzed GnRH-1 migration in Gli3$^{Xt/Xt}$ mouse mutants. In these mutants, we observed a dramatic reduction in the number of GnRH-1ns able to migrate form the nasal area into the brain. In Gli3$^{Xt/Xt}$ mutants, GnRH-1ns were found proximal to the vomeronasal organ forming tangles with a subset of Peripherin positive fibers, which were found to extend as far as the forebrain junction, where they also tangle in response to the lack of olfactory bulbs. Our data suggests that the loss of function of the Gli3 gene impairs the formation of the GnRH-1 migratory scaffold and GnRH-1 migratory ability. These observations indicate that Gli3 is a candidate gene for the etiology of HH.

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Table of Contents

Introduction

GnRH-1 neurons are a fundamental part of the hypothalamic-pituitary-gonadal (HPG) axis, controlling sexual development and reproductive hormone development (Paolo E Forni & Wray, 2015). GnRH-1 neurons travel from the vomeronasal organ (VNO) found in the olfactory pit to the pre-optic area of the basal forebrain where they release signals to the hypothalamus allowing the secretion of hormones. Luteinizing hormone (LH) and follicle stimulating hormone (FSH) secretion is elemental for reproductive function. In the ovary, LH produces estrogen and in the testis, LH produces testosterone. In males and females, FSH is necessary for the maturation of gametes (Paolo E Forni & Wray, 2015). Therefore, defective migration of GnRH-1 neurons from the olfactory pit results in hypogonadotropic hypogonadism (HH); HH is responsible for adverse reproductive development. Difficulty perceiving odors (hyposmia) or the lack of ability to smell (anosmia) associated with HH is clinically diagnosed as Kallman Syndrome (KS).

Point mutations in Gli3 have been identified in humans affected by HH (Vaaralahti et al., 2012). Gli3 acts as a transcriptional activator or repressor, mediated by the sonic hedgehog (Shh) signaling pathway. When Shh is present, it binds to its membrane-bound receptor, Patched-2, which then binds to a membrane-bound molecule, Smoothened, activating the Shh pathway. This mechanism prevents the phosphorylation and proteolytic cleavage of Gli3, and Gli3 acts as a transcriptional activator. To study the potential role of aberrant Shh signaling pathway and a nonfunctional Gli3 protein on GnRH-1 neuronal migration, we used a Gli3$^{Xt/Xt}$ mutant mouse model. Gli3$^{Xt/Xt}$ mouse mutants have an intragenic deletion of the *Gli3* gene that results in a loss of function of GLI3. Gli3$^{Xt/Xt}$ mice exhibit a plethora of severe phenotypes

including malformations of the brain, absence of the olfactory bulbs, and extra digits (Extra toe (Xt)) (Vaaralahti et al., 2012).

It is commonly believed that the migratory scaffold upon which the GnRH-1ns migrate is formed by axons of neurons of the olfactory system. However, unpublished data generated in Forni lab suggest otherwise. In fact, in Arx-1 null mice, a mouse model that lacks the olfactory bulbs and olfactory connection to the brain (Fulp et al., 2008), GnRH-1ns were found able to normally migrate into the brain along neuronal fibers of the putative terminal nerve (TN).

However, in the Gli3$^{Xt/Xt}$ mutants, we observed a complete loss of GnRH-1ns able to access the brain, and a reduction in the total number of GnRH-1ns in the whole animal. This suggests that the *Gli3* mutation affects the projections of the terminal nerve, disrupts normal GnRH-1 neuronal migration, and impairs normal GnRH-1 neurogenesis.

Gli3-null mutants do not survive past birth, as their severe brain malformations restrict that. On the other hand, Gli3 heterozygotes are viable and fertile. They survive past birth, occasionally with hydrocephaly, are able to reproduce, although some litters were found to be smaller than average, and have an average life span. Heterozygotes consistently have one extra toe and a small white spot on the belly in response to neural crest defects.

Materials and Methods

Animals Used

Gli3$^{Xt/Wt}$ mice were received from The Jackson Laboratory. The olfactory defects in Gli3$^{Xt/Xt}$ mice were previously characterized (Balmer & LaMantia, 2004). Gli3$^{Xt/WT}$ heterozygotes on a C57 Black genetic background were mated. Animals were euthanized using CO_2, followed by cervical dislocation. All animal procedures were done

in accordance with procedures approved by the University at Albany Institutional Animal Care and USE Committee (IACUC).

Tissue

Embryos were collected from time mated Gli3$^{Xt/Wt}$ females where a copulation plug was considered embryonic day 0.5 (E0.5). Embryos were fixed in 3.7% formaldehyde/PBS overnight then washed in PBS to remove the fix. Embryos were cryoprotected in 30% sucrose in PBS solution overnight. Embryos were embedded in O.C.T (Tissue-Tek) that was frozen with dry ice and stored in -80°C. Samples were cryosectioned using LEICA CM3050 S cryostat and collected on Superfrost plus slides (VWR) at 14 µm.

Genotyping Mice/PCR

Genotypes of mice were determined first by visual observation of multiple toes and confirmed doing PCR using the following primer sequences: wildtype forward primer 5'-GGCCCAAACATCTACCAACACATAG-3'; wildtype reverse primer 5'-GTTGGCTGCTGCATGAAGACTGAC-3'; mutant forward primer 5'-TACCCCAGCAGGAGACTCAGATTAG-3'; mutant reverse primer 5'-AAACCCGTGGCTCAGGACAAG-3'. Amplification products were analyzed by agarose gel electrophoresis.

Immunohistochemistry (IHC)

Sections were first incubated in and 30% hydrogen peroxide solution for 30 minutes. Sections were put into blocking solution (10% Horse Serum, 1.1% BSA, 0.1% Sodium Azide, 0.5% Triton in 200 mL 1x PBS) for 1-2 hours. Sections were incubated in primary antibodies overnight. The primary αbodies that were used are rabbit (Rb) α-Peripherin (1:500, Millipore Bioscience Research Reagents), SW rabbit (Rb) α-GnRH-1 (1:6000, Susan Wray, NIH), rabbit (Rb) α-active caspace-3 (1:1000, Millipore Bioscience Research Reagents). After primary antibodies, sections were incubated in secondary

antibody for 1-2 hours. The secondary antibody that was used is a biotinylated Donkey-α-Rabbit (Jackson Labs, 1:500). Sections were incubated in a horse-radish peroxidase detection kit (Vector Labs) for 1 hour. Staining was detected using a DAB solution that contained glucose and glucose oxidase and counterstained with methyl green for 30 seconds. Sections were mounted using Sub-X mounting (EMS). Epiflourescence and brightfield pictures were taken on a Leica DM4000 B LED fluorescence microscope equipped with a Leica DFC310 FX camera. Images were further analyzed using FIJ/ImageJ software.

Immunofluorescence (IF)

Immunoflourscent staining was preformed the same way as IHC with the exception of the initial hydrogen peroxide solution incubation. The primary antibodies that were used are chicken (Chk) α-Peripherin (1:1500, Abcam), Rabbit (Rb) α-GnRH-1 (1:6000, Susan Wray, NIH), goat (Gt) α-Neuropilin-2 (1:3000, R&D Systems), and goat (Gt) α-Olfactory Marker Protein (OMP) (1:4000, WAKO). Antigen retrieval was performed in a citric acid solution prior to incubation with chicken α-Peripherin. After primary antibodies, sections were washed in 1x PBS and incubated in the secondary antibody for 1½ hour. Species appropriate secondary antibodies were conjugated with Alexa-488 and Alexa-594 (Molecular Probes and Jackson Laboratories). Sections were counterstained with DAPI (4', 6' –diamidino-2-phenylindole; 1:3000, Sigma-Aldrich) for 3 minutes, dried, and cover slipped with Fluoro Gel (Electron Microscopy Services). Confocal microscopy pictures were taken on a Zeiss LSM 710 microscope. Epiflourescence pictures were taken on a Leica DM4000 B LED fluorescence microscope equipped with a Leica DFC310 FX camera. Images were further analyzed using FIJ/ImageJ software.

### Quantification and statistical analyses of microscopy data

Cell counts were done on serial sections immunostained for GnRH-1 and visualized under epiflourescence illumination (10X, 20X, 40X; Leica DM4000 B LED), according to their anatomical location [*i.e.*, (1) nasal area (VNO, axonal tracks surrounding the olfactory pits); (2) forebrain junction (FBJ); and (3) brain]. For each animal, counts were performed on 2 non-serial series. The average number of cells from these 2 series was then multiplied by the total number of series per animal to compute a value for each animal. These were then averaged (± standard error) among animals of the same age and genotype. Means ±SEs were calculated on at least two animals per genotype.

To generate GnRH-1 cell counts based on distance from the VNO, we measured the distance from the center of the VNO to the base of the basal forebrain subdividing the nasal area into 150-micron intervals. GnRH-1ns (GnRH-1 neurons) were counted in each interval, with any GnRH-1ns in the brain counted towards 'brain'.

Results

Ptch-2 is expressed along the GnRH-1 migratory tract

Hedgehog (Hh) functions by binding its transmembrane receptor proteins, Patched-1 (Ptch-1) and Patched-2 (Ptch-2). By analyzing the publically available images
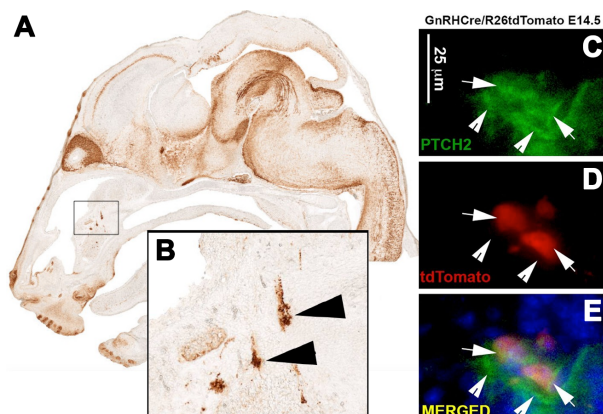

Figure 1. (A) Image taken from GENSAT website of Ptch-2 EGFP BAC transgenic (B) Blow up of area indicated in (A) showing EGFP positive cell bodies (black arrow heads) in the migratory tract (C,D,E) IF staining against Ptch-2 (green) and tdTomato (red) on a GnRHCre/R26tdTomato and DAPI (blue). Co-localization of Ptch-2 and tdTomato positive cell bodies (white arrows). Ptch-2 positive fibers (white arrow heads).

of the BAC transgenic reporters generated by the GENSAT consortium (IHC available on gensat.org), we identified that Ptch-2 is expressed by cells along the GnRH-1 migratory pathway (Fig.1A,B). Performing an immunofluorescent staining against Patched-2 on GnRHCre/R26tdTomato reveled Patched-2 expression in the GnRH-1 neurons and along the fibers of the terminal nerve (TN).

GnRH-1 neurons do not access the brain in Gli3$^{Xt/Xt}$ mutants

GnRH-1 neurons are generated in the VNO between embryonic day 10.5 and 11.5 (E10-11.5) and finish their migration into the brain during embryonic development by E18.5 (P. E. Forni, Bharti, Flannery, Shimogori, & Wray, 2013; Jasoni, Porteous, & Herbison, 2009). To investigate whether a disrupted Gli3 protein, which is downstream of Shh and Ptch2 signaling, can disrupt GnRH-1 neuronal migration we utilized Gli3$^{Xt/Xt}$ mutants at E13.5 and E15.0.
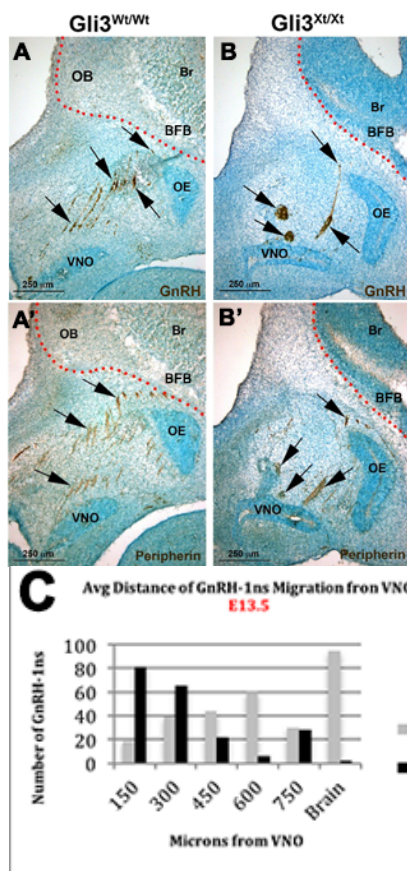
E13.5 and E15.0 sections were immunostained against GnRH-1. In the E13.5 wildtype (WT), the majority of the GnRH-1ns were found in the nasal area (NA), while the expected one-third of the population was found in the brain (Fig.2C). In the E13.5
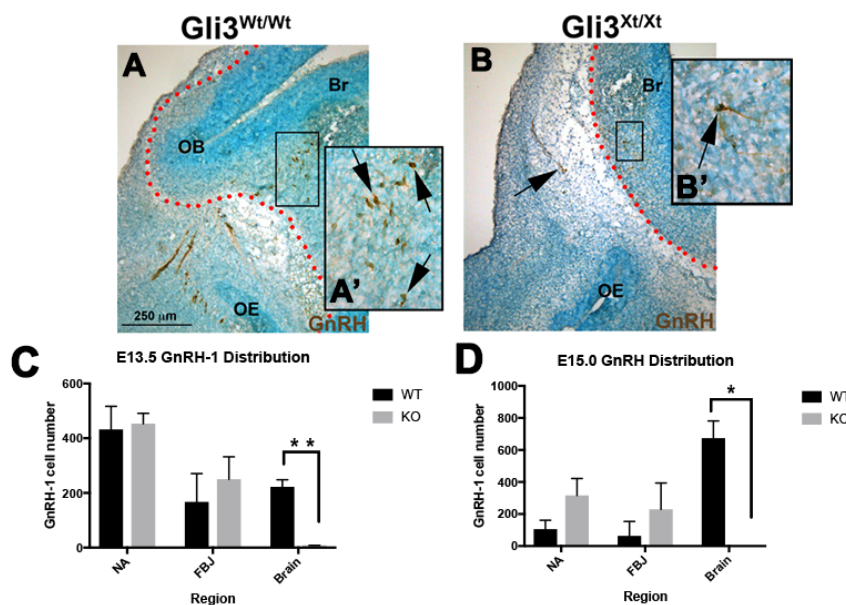
Figure 2. IHC against GnRH on E13.5 WT (A) and mutant (B) Blow up of area indicated in (A) showing GnRH-1ns in the brain (arrows) in the WT (A') and the mutant (B') GnRH counts at E13.5 (C) and E15.0 (D)

mutant mice, we found that 64% of the total GnRH-1ns (n=2) were stuck proximal to the VNO as compared to 43% in the WT. Interestingly there was a dramatic reduction in the number of GnRH-1ns able to access the brain in the mutants (Fig 2B and D). There was a 23% reduction of the total GnRH-1ns, as well, (n=2) (Fig.2A and C) indicating either cell death or a reduction in neurogenesis. At E15.0, we see a similar phenotype to E13.5, whereas in the WTs, we see GnRH-1ns accessing the brain, no GnRH-1 neurons can be found in the brain of the mutant. (Fig.2D). We also observed that 59% of GnRH-1ns were stuck in the NA compared to 12% in the WT, with a 38% total loss of GnRH-1ns in the animal (n=2) (Fig.2D).

GnRH-1ns and its migratory scaffold get stuck proximal to the VNO

By measuring GnRH-1 neuronal migration from the VNO to the brain, we observed that the majority of the GnRH-1 neurons in the Gli3$^{Xt/Xt}$ mutants were unable to go further than 300μm from the VNO, whereas in the WT, GnRH-1ns were found as far as the brain in the both E13.5 and E15.0 (Fig.3C and D). Notably, a small group of GnRH-1 neurons was

Figure 3. IHC against GnRH on E13.5 WT (A) and mutant (B) IHC against Peripherin on E13.5 WT (A') and mutant (B') Arrows pointing to GnRH-1ns (A and B), and Peripherin (A' and B') GnRH neuronal migration from VNO measured in E13.5 WT and mutant (C) and E15.0 WT and mutant (D)

consistently found to behave differently from the GnRH-1ns stuck in the tangles proximal to the VNO. There is a distinct group of GnRH-1ns in the mutants that are found as far as 750um from the VNO, which is where the olfactory bulb (OB) is normally found (Fig.3C).

Peripherin is an intermediate filament expressed by the peripheral nervous system, which can highlight the terminal nerve (Fig. 4A). Gli3$^{Xt/Xt}$ mutants form Peripherin positive tangles near the VNO and in the FBJ (Fig.3B'), whereas in the WT, we see Peripherin positive fibers projecting towards the brain (Fig.3A'). Most of the GnRH-1ns form tangles proximal to the VNO (Fig3 C, D), which mirrors the Peripherin positive tangles. This suggests that a nonfunctional Gli3 protein affects TN projection and GnRH-1 neuronal migration. However, Peripherin is not specific and also highlights vomeronasal and olfactory fibers.
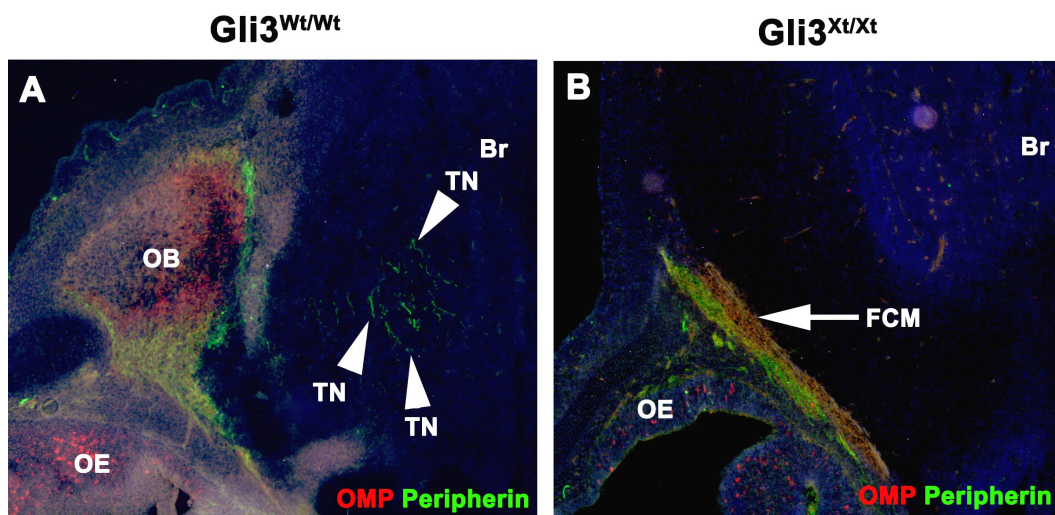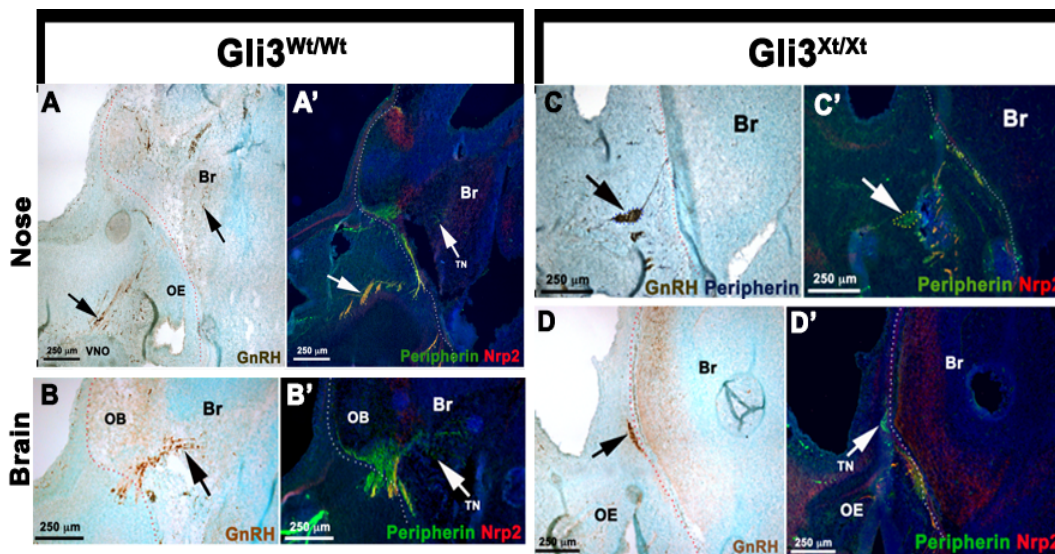

Figure 4. IF staining against Peripherin (green) and OMP (red) on E15.0 WT (A) and mutant (B) Arrow heads point to TN (A) Arrows point to fibrocellular mass (FCM) (B)

To differentiate between olfactory and TN projections we did an IF staining for olfactory marker protein (OMP), which highlights olfactory neurons and their projections, and Peripherin on E15.0 WT and mutant sections. The TN can be seen in the WT as OMP negative, Peripherin positive and accessing the brain (Fig.4A), while in the KO

there are no Peripherin positive fibers accessing the brain and no distinction in the fibrocellular mass (FCM) between Peripherin only and OMP only fibers (Fig.4B).

In order to assess whether GnRH-1ns are actually tangling on the TN, we used Neuropilin-2 (Nrp2), a known marker for vomeronasal projections (Casoni et al., 2016), to differentiate between the TN and vomeronasal fibers. Under WT conditions, in the NA we see that GnRH-1ns are migrating towards the brain on Nrp2 positive, Peripherin positive TN bundles (Fig.5A and A'). However, we observed that the GnRH-1ns access the brain on Peripherin positive, Nrp2 negative fibers (Fig.5B and B'), which is indicative of the TN. When we look at the mutants, we see GnRH-1 neuronal tangles in the nasal area on Peripherin positive, Nrp2 negative fibers (Fig.5C and C') suggesting that GnRH-1ns get stuck on the putative TN and not on vomeronasal fibers. We see something similar in the FBJ of mutants, where GnRH-1ns are stuck on Peripherin positive, Nrp2 negative fibers (Fig5.D and D'). This further confirms that the TN is genetically distinct from olfactory and vomeronasal fibers. This data suggests that the Gli3 mutation and

Figure 5. IHC against GnRH on E15.0 WT (A and B) and mutant (C and D) IF against Peripherin (green) and Nrp2 (red) on E15.0 WT (A' and B') and mutant (C' and D') Arrows point to GnRH-1ns in A,B,C,D. TN represented by arrows pointing to Peripherin positive, Nrp2 negative fibers in A',B',C',D'

13

impaired Shh signaling affects the TN which plays a major role in GnRH-1 neuronal migration.
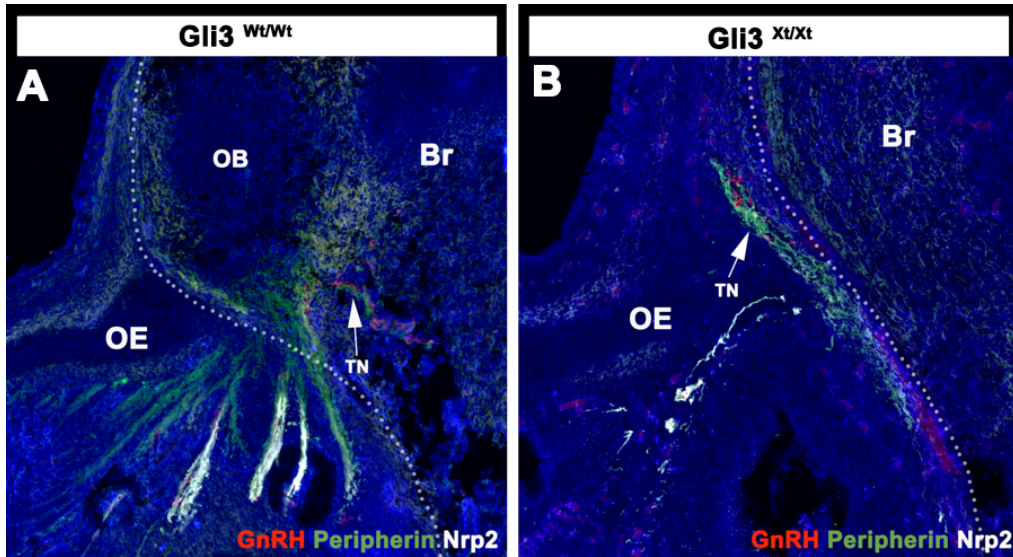

Figure 6. Confocal images of E15.0 WT (A) and mutant (B) immunostained against GnRH (red), Peripherin (green), and Nrp2 (white) Arrows point to TN

A triple immunostaining against GnRH, Peripherin, and Nrp2 done mostly to recapture our previous observations that GnRH-1ns travel on the Peripherin positive, Nrp2 negative TN (Fig6A,B) further confirms our hypothesis. In the mutant, we observed the GnRH-1ns getting stuck in the FBJ on Peripherin positive, Nrp2 negative fibers, indicating that the TN cannot enter the brain, forbidding GnRH-1ns from accessing the brain (Fig.6B).

The Terminal nerve has multiple branches

Under normal conditions, it has been recognized that a subset of GnRH-1ns form a 'ring of GnRH' around the olfactory bulb (OB) (Casoni et al., 2016). Although we do not consistently see such a neat streak of GnRH-1ns (Fig. 7B), we constantly observe a few GnRH-1ns that make it further up the brain than the remaining bundle of GnRH-1ns in the mutants (Fig.3 C-D). Interestingly enough, this is where the olfactory bulb (OB) is normally found. We found that these GnRH-1ns are, as well, stuck on Peripherin positive, Nrp2 negative fibers (Fig.6B). This may suggest that there is a branch of the terminal nerve that, without fail, chooses this specific route, along with a subset of GnRH-1ns that, accordingly, migrate along those fibers around the OB. In the mutant mouse, we observed that this possible TN branch is still present and consistently takes this alternate route. In addition, it is always accompanied by a subset of GnRH-1ns that migrate along this putative branch of the TN (Fig.7B).
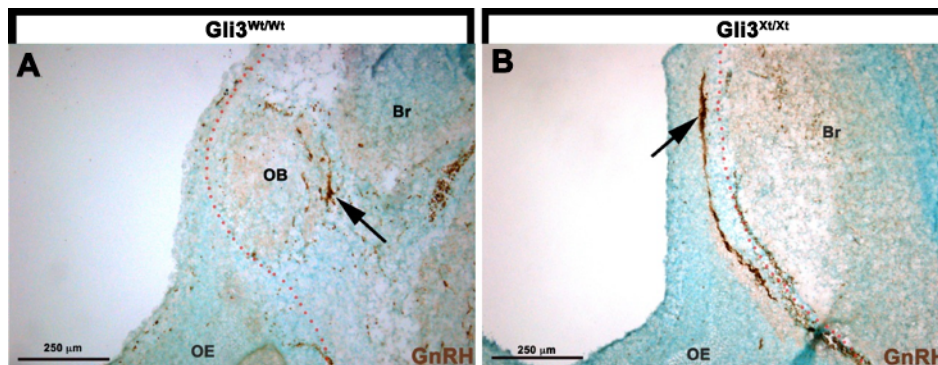
Figure 7. IHC against GnRH on E15.0 WT (A) and mutant (B) Arrows point to GnRH-1ns showing GnRH-1ns surrounding OB (A)

IF staining against Peripherin and Nrps2 highlights the two branches of the TN diverging, where one is diving into the pre-optic area (POA) of the basal forebrain while the other is projecting toward the OB (Fig.8A). IHC staining against GnRH shows two populations of GnRH-1ns projecting to different areas of the brain on the differentiating branches of the putative TN (Fig.8B).
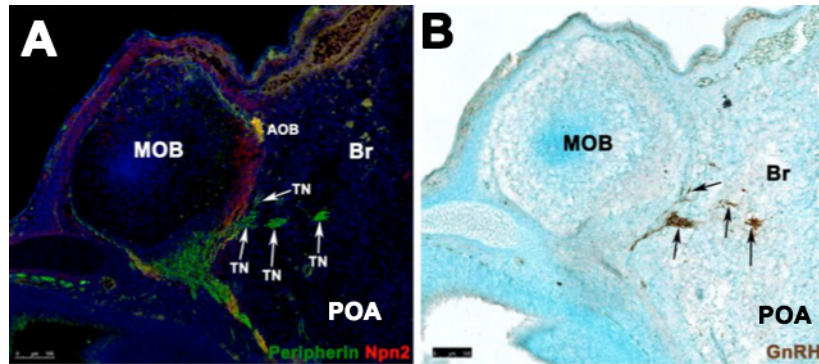
Figure 8. IF staining against Peripherin (green) and Nrp2 (red) (A) and IHC staining against GnRH (B) on E15.5 WT embryo. Arrows point to the two branches of the diverging TN (A) and the GnRH-1ns migrating on the TN (B)
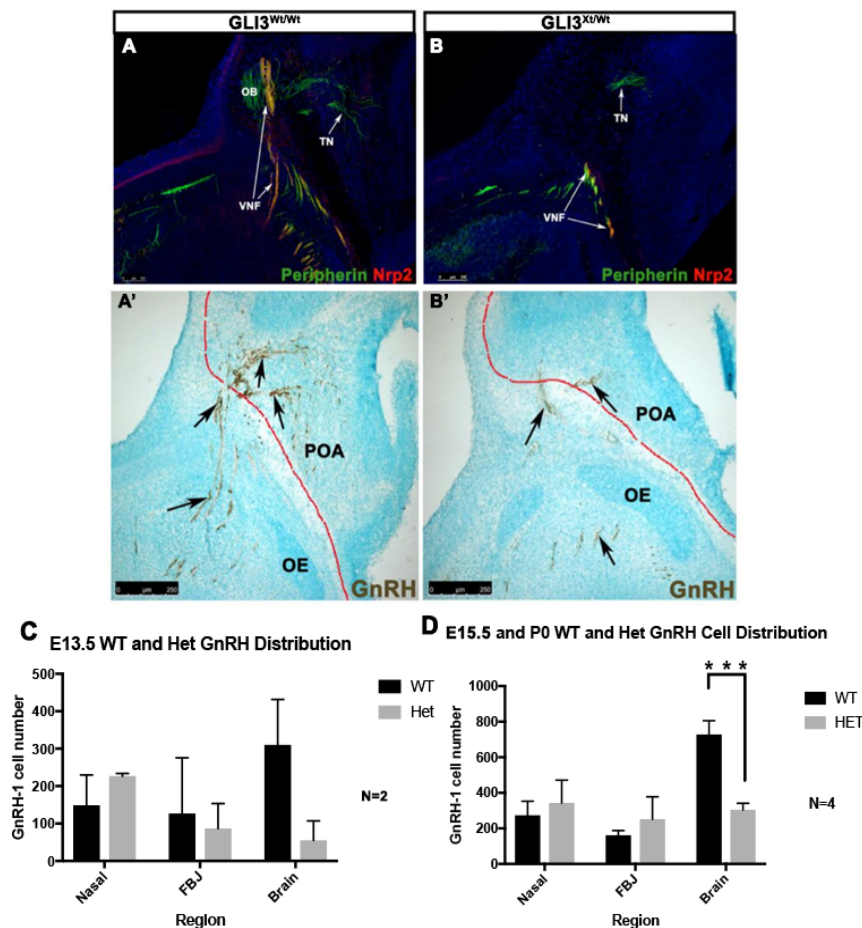
Gli3$^{Xt/Wt}$ Heterozygotes show haploinsufficiency

We were interested in studying GnRH-1 migration in heterozygotes because they show a general phenotype of an extra toe. Homozygous mutants show no GnRH-1 neuronal access to the brain, yet heterozygotes are viable and fertile, suggesting their anatomy to mirror a WT. As expected, GnRH-1ns do enter the brain, however, it is a reduced amount, an amount that is just enough to allow fertility. The reduced amount of GnRH-1ns that access the brain in heterozygotes could be the cause of smaller litters and slower weight gain during pregnancy.

Most noticeably, the TN in the heterozygotes enters the brain, but is shorter and less branched (Fig.9B). In the WT, chemoattractants and repellants direct the projection of the TN into the pre-optic area (POA) of the basal forebrain, which allows the migration of the GnRH-1ns into the POA (Fig.9A and A'). In the heterozygote, the TN does not

extend into the POA and the GnRH-1ns just make it into the brain but do not reach the POA (Fig.9B and B'). From our GnRH-1 cell quantifications, we observed a total number of GnRH-1ns that is similar to the WT. However, there was a significant reduction of GnRH-1ns that entered the brain (Fig.9D), further confirming that heterozygotes show an intermediate phenotype, or haploinsufficiency.

To confirm if this phenotype is a real result, we looked at postnatal day zero (P0) Gli3$^{Xt/Wt}$ heterozygote mutants. Due to animal mating issues, we were unable to get ample embryos to complete an n=3 for all ages we tested. As a majority of GnRH-1ns finish their migration into the POA by E16.5 (P. E. Forni, Bharti, Flannery, Shimogori, & Wray, 2013), and since we saw that the P0 GnRH-1 cell counts were similar to our E15.5 heterozygous counts, we were able to combine our E15.5 and P0 counts to test for any significant difference. With an n=4 for

Figure 9. IF staining against Peripherin and Nrp2 on E13.5 Gli3$^{Wt/Wt}$ (A) and Gli3$^{Xt/Wt}$ (B) IHC staining against GnRH on E13.5 Gli3$^{Wt/Wt}$ (A') and Gli3$^{Xt/Wt}$ (B') White arrows point to vomeronasal fibers (VNF) and the TN (A and B) Black arrows point to GnRH-1ns (A' and B') Cell counts of E13.5 Gli3$^{Wt/Wt}$ and Gli3$^{Xt/Wt}$ (Nasal P=0.306; FBJ P=0.761; Brain P=0.112) (C) and E15.5 & P0 Gli3$^{Wt/Wt}$ and Gli3$^{Xt/Wt}$ (Nasal P=0.582; FBJ P=0.426; Brain P<0.0001) (D)

heterozygous mutants, our t-test evaluation showed that there was a significant reduction of GnRH-1ns accessing the brain (P<0.0001) (Fig.9D).

Interestingly, at E13.5, our results were variable. We saw less total GnRH-1ns than we did in mutants of the same age. However, we saw no significant difference between the WT and the heterozygote; this could be due to loss of sections.

Discussion

After characterizing two different ages, E13.5 and E15.0, we can conclude that an impaired Shh signaling pathway and a nonfunctional Gli3 protein causes severe developmental delay and defected GnRH-1 neuronal migration. Interestingly, we see an overall reduction in the number of GnRH-1 neurons but did not see a difference in cleaved-caspase-3, a marker for cell death, (Data not shown n=1) but this will need to be further characterized, as no quantifications were made. However, we did notice a reduction in the volume of the olfactory epithelium (OE) (Data not shown n=1), which may be due to a developmental delay. In an immunostaining against OMP, we confirmed that the reduction in OE volume resulted in a decreased number of mature olfactory neurons (OMP+ cells) in the OE. This further suggests that the Gli3 mutation either affects neurogenesis or differentiation of neurons into mature olfactory neurons.

Our stainings also confirm that GnRH-1ns enter the brain on Peripherin positive fibers that are also negative for Nrp2 (vomeronasal fiber marker) and OMP (olfactory fiber marker). In the mutants, we see GnRH-1ns forming tangles only on Peripherin positive fibers near the VNO and in the FBJ. This suggests to us that the GnRH-1ns migration is affected by the *Gli3* mutation since both the TN and the GnRH-1ns are aberrant. One of our future goals is to find a marker for the TN. This will allow us to accurately depict the effects of a nonfunctional GLI3 and an impaired Shh signaling

pathway on TN projections. One potential marker we are currently testing is Calbindin, which is a calcium binding protein. We see that Calbindin is expressed along the migratory route of the GnRH-1ns. Thus far, GnRH-1ns seem to be negative for Calbindin, suggesting the expression may be highlighting the probable TN.

In the Gli3$^{Xt/Wt}$ embryos, an intermediate phenotype between the WT and mutant was observed; that led us to conclude that this is a phenomenon of haploinsufficiency, where one copy of the Gli3 gene does not produce enough of the Gli3 protein to mirror the wild type phenotype. We see with Peripherin IF that the TN is unable to fully branch and dive into the POA of the basal forebrain, and also, interestingly see a reduction in the number of GnRH-1ns in the brain. This could suggest that Gli3 may affect the fine targeting of the TN which may alter the GnRH-1 neuronal migratory route, which may causes HH in humans. This may explain the slow weight gain of pregnant Gli3$^{Xt/Wt}$ mice and the small litters we collect with Gli3 matings. Although the E15.5 heterozygote counts mirrored our expectations, one thing we need to investigate in the future is why there was a total decrease in GnRH-1ns in the E13.5 heterozygotes, even compared to the E13.5 homozygous mutants. Though when put through a t-test, the overall number of GnRH-1ns is seen as an insignificant difference (n=2) (Nasal P=0.306; FBJ P=0.761; Brain P=0.112). This we believe is the result of lost sections during sectioning, so we will need to increase our sample size and exclude some of the previous data.

The heterozygote characterizations also confirm the connection between Gli3 and Shh and its effects on the migration of GnRH-1ns and the projections of the TN. Heterozygotes are missing one functional Gli3 gene and have less of the Gli3 protein, which naturally acts as a transcriptional repressor, so we may be seeing an overexpression of downstream genes. Previous literature has shown how Shh signaling regulates neuropilin-1 (Nrp1) expression (Eisner et al., 2015), a receptor for a

chemorepellant, which is expressed by the TN. This may tell us that the Gli3 gene and the Shh pathway play a large role in projecting the TN to the correct area and directing the migration of GnRH-1ns. With one copy of the Gli3 gene, there are still enough GnRH-1ns accessing the brain to allow for sexual development, but we would like to observe whether the heterozygous mutants go through puberty at later stages. This condition mirrors the phenotype of KS patients, individuals presenting with HH and anosmia. KS patients undergo a continuum of phenotypes. Some go through various degrees of puberty at different ages, while others don't undergo puberty at all and are infertile. We will continue to study Gli3 mutants to better understand the KS phenotype, while using the Gli3 heterozygotes to depict the affects of a nonfunctional Gli3 protein and a defective Shh signaling pathway on TN projections and GnRH-1 neuronal migration.

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