Título de la Tesis

DISRUPTION OF ZEBRAFISH *PROKINETICIN2* SIGNALING CAUSES KALLMANN SYNDROME RELATED PHENOTYPES

Tesis entregada a la

UNIVERSIDAD DE VALPARAISO

En cumplimiento parcial de los requisitos para optar al grado de Doctor en Ciencias con Mención en Neurociencias.

Facultad de Ciencias

Por

Joaquín Letelier Undurraga

Marzo, 2012

Tutor: **Dra. Kathleen Whitlock**

FACULTAD DE CIENCIAS

UNIVERSIDAD DE VALPARAÍSO

INFORME DE APROBACIÓN DE TESIS DE DOCTORADO

Se informa a la Dirección de Postgrado de la Facultad de Ciencias que la tesis del Doctorado presentada por el candidato:

Joaquín Letelier Undurraga

Ha sido aprobada por la comisión de Evaluación de la Tesis como requisito para optar al grado de Doctor en Ciencias con Mención en Neurociencias, en el examen de defensa de Tesis rendido el 05 de Marzo de 2012.

DIRECTOR DE TESIS:

Dra. Kathleen Whitlock.....Tutor

COMISIÓN DE TESIS:

Dr. John Ewer..... Presidente

Dr. Miguel Allende.....

Dr. Agustín Martínez.....

Dr. Juan Larraín.....

Acknowledgements.

Luego de casi 6 años realizando mi doctorado es muy difícil agradecer a toda la gente que me ayudó durante este largo tiempo y tampoco quiero que esta sección sea larga y aburrida. Aún asi, no puedo dejar de nombrar a mis más cercanos compañeros de laboratorio (Juanita, Eileen, Francisco, Katica, Sole, Rodrigo, Felipe, Mauricio) que me ayudaron en innumerables tareas y también a grandes profesores que encontré en el camino (Oliver S., David N., Agustín M., Miguel A., Christian B., Pablo M., Patricio V.) que supieron guiarme en los momentos adecuados. Agradezco de manera profunda a todos los integrantes nuevos y antiguos del CINV (Carola, Vivian, Isaac, Angie, Pedro, Alvaro, Claudio,….que difícil nombrarlos a todos….) por todo el apoyo y la buena disposición constante.

Especialmente quiero agradecer a Kate y a John por todo lo entregado (académica, profesional y personalmente hablando) durante tanto tiempo. Gracias miles.

Finalmente y no menos importante agradezco a mi familia y amigos que a pesar de no entender nunca ni una mínima pizca de lo que se trató mi investigación (a pesar del esfuerzo por hacerlo), siempre estuvieron ahí desinteresadamente para apoyarme, preguntarme e interesarse por mi trabajo.

MUCHAS GRACIAS A TODOS!

Financial support.

This PhD thesis was supported by:

- Proyecto FONDECYT 1111046;
- Proyecto FONDECYT 1071071;
- NIH/NICHD-050820 Grant;
- Mideplan-ICM CGC nucleus;
- Mideplan-ICM CINV institute;
- Beca MECESUP para estudios de doctorado en Chile;
- Beca CONICYT para estudios de doctorado en Chile;
- Beca CONICYT para asistencia a congresos nacionales;
- Beca CONICYT para asistencia a congresos en el extranjero;
- Beca MECESUP para asistencia a congresos en el extranjero;
- Beca CONICYT para término de tesis doctoral.
- Beca MECESUP para término de tesis doctoral.

Table of contents.

List of figures and tables.

Abbreviations

AUG: mRNA translational start site.

BLAST: Basic local alignment sequence tool.

BSA: Bovine serum albumin.

CNC: Cranial neural crest.

CNS: Central nervous system.

DAB: Diaminobenzidine.

DEPC: Diethylpyrocarbonate.

DIG: Digoxigenin.

dpf: Days post fertilization.

DPX: distyrene plasticizer xylene medium

Emx1: *Empty spiracles homeobox 1* gene.

Fgfr1: *Fibroblast growth factor receptor 1* gene.

FSH: Follicle-stimulating hormone.

GFP: Green fluorescent protein.

GnRH: Endocrine gonadotrophin-releasing hormone.

GnRHR1: GnRH receptor 1.

GPCR: G protein-coupled receptors.

H-GnRH: Hypothalamic GnRH.

HH: Hypogonadotrophic hypogonadism.

HiTE: High tris EDTA buffer.

hpf: Hours post fertilization.

HPG: Hypothalamic-pituitary-gonad axis.

HSPGs: Heparan sulfate proteo-glycans.

Hv: Ventral zone of the periventricular hypothalamus.

IHH: Idiopathic hypogonadotropic hypogonadism.

ISH: *In situ* hybridization.

KAL1: X -linked KS caused by *anosmin 1* mutations.

Kal1a: zebrafish *kallmann 1a* gene.

Kal1b: zebrafish *kallmann 1b* gene.

KAL2: autosomal dominant KS caused by *fgfr1* mutations.

KO: knock-out.

KS: Kallmann syndrome.

LB: Lysogeny broth.

LH: Luteinizing hormone.

MHB: Midbrain hindbrain boundary.

mm: Mismatch control morpholino.

MOs: Morpholinos.

NCCs: Neural crest cells.

nIHH: Normosmic IHH.

Nwt: New wild-type strain.

OB: Olfactory bulb.

OE: Olfactory epithelium.

OMP: Olfactory marker protein.

OP: Olfactory placode.

OSNs: Olfactory sensory neurons.

OT-like: *Oxytocin-like* gene.

Otp: *Orthopedia* gene.

Otx2: *Orthodenticle* 2 gene.

PBDT: Phosphate BSA DMSO Tween buffer

PBS: Phosphate buffered saline.

PFA: Paraformaldehyde.

POA: Preoptic area.

POMC: *proopiomelanocortin* gene.

PROK1: Prokineticin 1 protein.

Prok2: *prokineticin2* gene.

PROKR1: Prokineticin receptor 1 protein.

Prokr2: *prokineticin receptor2* gene.

qPCR: Quantitative real-time PCR.

RT: Room temperature.

RT-PCR: Reverse transcriptase polymerase chain reaction.

Shh: *Sonic hedgehog* gene.

SSC: Saline sodium citrate buffer.

TAE: Tris acetate EDTA buffer.

TN: Terminal nerve.

tRNA: Torula yeast RNA.

WAB: whole adult brain expression.

YFP: Yellow fluorescent protein.

Zv9: Ninth released version of the zebrafish genome.

RESUMEN

En una gran cantidad de organismos, la hormona liberadora de gonadotropinas juega un rol crucial en el establecimiento del eje hipotálamo-pituitaria-gónadas (HPG). En humanos, mutaciones en una variedad de genes resultan en una enfermedad llamada síndrome de Kallmann (KS), la cual se caracteriza por presentar individuos anósmicos (sin sensación olfativa) y con hipogonadismo (gonadas pequeñas) hipogonadotrópico (baja concentración de gonadotropinas en el suero sanguíneo). Este síndrome está causado por la falta de las células endocrinas de GnRH presentes en el hipotálamo. En estudios recientes se encontró que los genes *prokineticina 2* (*prok2*) y el *receptor de prokineticina 2* (*prokr2*) están involucrados en el desarrollo del bulbo olfatorio y del sistema reproductor y que mutaciones en cualquiera de estos genes generan fallas fenotípicamente similares a las observadas en los pacientes con KS. Usando como modelo de estudio al pez cebra, nosotros investigamos el rol de los genes *prok2* y *prokr2* en el desarrollo embrionario del hipotálamo, la glándula pituitaria y el sistema olfatorio, ya que estos tejidos están intimamente ligados durante el desarrollo temprano del embrión. A partir de la secuencia de los genes *prok2* y *prokr2* de ratón, nosotros identificamos genes en el genoma del pez cebra (Zv9) que codifican para proteinas con un alto grado de identidad en relación a las proteinas PROK2 y PROKR2 de mamífero. En estos análisis encontramos dos genes homólogos a *prokr2* de ratón, llamados *prokr2a* y *prokr2b*, los cuales presentan un 69% y un 62% de identidad a nivel proteico. Además encontramos un gen homólogo a *prok2*, el cual presenta un 65% de identidad aminoacídica.

Mediante RT-PCR demostramos que estos genes se expresan en embriones y en el cerebro adulto de pez cebra. Además, utilizando sondas específicas para hibridación *in situ*, observamos que el gen *prok2* está expresado en la región hipotálamica del cerebro adulto. Para disminuir la función de los genes *prokr2a*, *prokr2b* y *prok2* utilizamos morfolinos específicos y examinamos el efecto de esta pérdida de función en el desarrollo de tejido hipotalámico, olfatorio y de la pituitaria anterior. La inyección de estos morfolinos causa una reducción significativa en el número de células de GnRH endocrinas y en el número de células similares a *oxitocina* (ambas hipotálamo específicas) en comparación con el control. Además, utilizando la linea transgénica POMC:GFP encontramos que la pituitaria anterior se forma de manera anormal en los embrios inyectados con morfolinos. Por último encontramos que la pérdida de función de los genes *prok2*, *prokr2a* y *prokr2b* altera el patrón esteriotipado de terminación axónica de las neuronas sensoriales olfatorias en el bulbo olfatorio (OB) y que este último además está disminuido en tamaño. Correlacionado con este defecto, encontramos que la expresión del factor de transcripción *emx1* está ausente en el OB de los embriones tratados con morfolinos. En conclusión, demostramos que el pez cebra posee genes homólogos a *prok2* y *prokr2* de ratón y que la pérdida de función de estos genes resulta en un desarrollo anormal del sistema olfatorio y el hipotálamo, lo cual recapitula el fenotipo y las características clínicas del KS.

Palabras clave: Células GnRH endocrinas, hipogonadismo hipogonadotrópico, síndrome de Kallmann, *prokineticina 2*, hipotálamo, sistema olfatorio.

ABSTRACT

Endocrine gonadotrophin-releasing hormone (GnRH) cells play a crucial role in the establishment of the hypothalamic-pituitary-gonad (HPG) axis. Mutations in a variety of genes result in Kallmann Syndrome (KS) characterized by anosmia (loss of sense of smell) and hypogonadotrophic hypogonadism (HH) which is caused by the lack pulsatile release of endocrine GnRH from the hypothalamus. Recent studies have found that the *prokineticin2* (*prok2*) and *prokineticin receptor2* (*prokr2*) genes are involved in the development of olfactory bulb and hypothalamus, and mutations in either of these genes result in a phenotype similar to KS. Using zebrafish as a model system we have investigated the role of the *prok2* and *prokr2* genes in the development of hypothalamic, pituitary, and olfactory tissues. We identified zebrafish homologues of the mouse *prok2* and *prokr2* genes. We found that zebrafish have two homologues of the mouse *prokr2* gene, *prokr2a* and *prokr2b*, with 69% and 62% identity at the amino acid respectively. In contrast, we found only one homologue of *prok2* mouse gene with a 65% identity at the amino acid level. Using RT-PCR analysis we showed that these genes are expressed in zebrafish larvae and adult brain tissues. In addition, *in situ* hybridization showed that the *prok2* gene is expressed in a restricted group of cells in the adult hypothalamus. To analyze *prokr2a*, *prokr2b* and *prok2* function, we used morpholinos (MO) to disrupt ("knockdown") gene function by affecting translation. The knockdown of *prokr2a*, *prokr2b* and *prok2* genes resulted in a significant reduction in the number of hypothalamic GnRH and *oxytocin-like* cells compared with controls. In addition, the anterior pituitary lacked the stereotypical pattern of

pro-opiomelanocortin expressing cells as assayed by POMC-GFP expression. Finally, we found that in the *prok2*, *prokr2a* and *prokr2b* knockdown embryos the axons of the olfactory sensory neurons did not form the stereotyped termination pattern in the developing olfactory bulb. Correlated with this defect, *emx1* expression was totally absent in the developing olfactory bulb. Here we show that zebrafish have homologues of the mouse *prok2* and *prokr2* genes and that knockdown of the *prok2*, *prokr2a* and *prokr2b* genes results in abnormal development of the olfactory system and hypothalamic cell types, a phenotype resembling the clinical manifestations of KS.

Key words: Endocrine GnRH cells, hypogonadotrophic hypogonadism, Kallmann syndrome, *prokineticin2*, hypothalamus, olfactory system.

1. INTRODUCTION.

1.1 GnRH functions and developmental origin.

Gonadotrophin-releasing hormone (GnRH) is a multifunctional decapeptide found in all vertebrates that plays endocrine as well as neuromodulatory roles. The different form of this peptide (endocrine and neuromodulatory) modulate reproductive behaviors, affect reproductive tissue via the hypothalamus (**Soga et al. 2005**), and have been proposed to regulate peripheral nervous systems (**Stell et al., 1987**; **Eisthen et al., 2000**; **Wirsing-Wiechmann et al., 2002**; **Kawai et al., 2009**). In mammals, endocrine GnRH-secreting cells comprise a small number of neurons that are found primarily in the preoptic area (POA) and the caudal hypothalamus. The pulsatile release of the hypophysiotrophic (which means that act on or stimulate the hypophysis) form of GnRH from the hypothalamus induces the release of gonadotrophins from the adenohypophysis, which in turn promotes the development of secondary sexual characteristics development in the gonads making this peptide essential for vertebrate reproduction.

In fish, there are three isoforms of GnRH (GnRH1, GnRH2, and GnRH3) but because all forms are not present in all fishes they are also named according to their location in the central nervous system (CNS). The form located in the hypothalamus is H-GnRH (GnRH1 or GnRH3), the form found in the midbrain tegmentum is midbrain-GnRH2 (always GnRH2), and the terminal nerve form is TN-GnRH (GnRH1 or GnRH3) (**Whitlock, 2005b**) (**Figure 1**). GnRH1 is the primary neuropeptide regulator of reproductive processes in vertebrates (**Mason et al.,**

1986). The isoform found in the midbrain, GnRH2, has been linked to modulation of reproductive behaviors and is characterized by its highly conserved amino acid sequence across vertebrates, from jawed fish to mammals (**Dubois et al., 2002**).

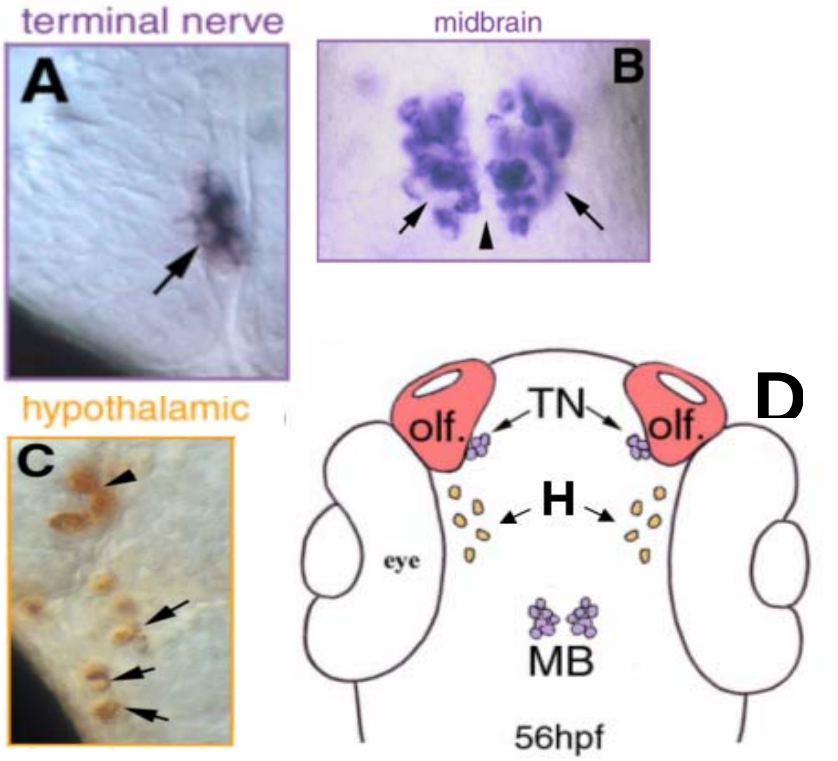


Figure 1. Summary showing distinct GnRH forms present in zebrafish embryos. (**A**) *In situ* hybridization showing TN-GnRH (or GnRH3) expression in the terminal nerve (black arrow). (**B**) *In situ* hybridization showing expression of GnRH2 in the midbrain (black arrows) with two clusters of cells lying in on either side of the midline (black arrowhead). The GnRH2 and TN-GnRH expressing cells arise from cranial neural crest (**Whitlock et al., 2003; Whitlock et al., 2005a**). (**C**) Immunohistochemistry showing GnRH expression in the ventral diencephalon of the embryos (black arrows) and terminal nerve (arrowhead). (**D**) Diagram showing the location of these three different forms of GnRH in a zebrafish embryo at 56hpf. TN: terminal nerve, H: Hypothalamic, MB: Midbrain. Cells indicated in purple are neuromodulatory in function and those in orange endocrine function. From **Whitlock et al., 2005a**.

The TN cells expressing GnRH (TN-GnRH) are proposed to act as modulator of olfactory behaviors (**Wirsing-Wiechmann et al., 2002**) and visual acuity (**Stell et al., 1987**). Both GnRH2 and TN-GnRH isoforms are proposed to

originate from cranial neural crest (CNC) (**Whitlock et al., 2003, Whitlock et al., 2005a**).

Using immunocytochemistry and *in situ* hybridization, two separate laboratories reported in 1989 that GnRH-positive cells in the ventral forebrain apparently arose from the olfactory placode (OP) and then migrated from the nasal septum into the forebrain via the terminal and vomeronasal nerves (**Schwanzel-Fukuda & Pfaff, 1989, Wray et al., 1989a, Wray et al., 1989b**). This was the first report of an endocrine cell type with a proposed embryonic origin in a sensory system. More recently, studies in chick (**el Amraoui & Dubois, 1993**), medaka (**Parhar et al., 1998**), zebrafish (**Whitlock et al., 2003**; **Whitlock, 2005b**), and mouse embryos (**Forni et al., 2011**) have suggested that these cells do not originate in the OP. In chick embryos, GnRH1 expressing cells are first apparent in the anterior limits of the neural fold (**Witkin et al., 2003**), which is a region that give rise to precursors of the anterior pituitary placode. In zebrafish embryos the regions that will form the OPs are at the anterior limit of the neural plate and flank the region that give rise to the anterior pituitary placode (**Whitlock & Westerfield, 2000**). The anterior pituitary precursors abutt the anterior border of the hypothalamic precursors along the midline of the anterior neural plate (**Karlstrom et al. 1999**). Thus the OP is closely associated with the region of the neural plate that will form the adenohypophyseal (anterior pituitary) placode and anterior hypothalamus (**Whitlock & Westerfield, 2000**) (**Figure 2**).

Using *you-too* (**Kalstrom et al., 1999**) and *detour* (**Kalstrom et al., 1996**) zebrafish mutants (*gli1* and *gli2* gene mutations, respectively), which show among other features total or partial loss of the anterior pituitary gland, Whitlock and

colleagues showed that these animals lack GnRH cells in the hypothalamus (**Whitlock et al., 2003**) and that this loss of hypothalamic GnRH cells is correlated with the loss of the anterior pituitary gland but not the OPs. Specifically they suggested that these cells emerge from precursors of the anterior pituitary placode which is consistent with the types of cells generated by this tissue. *gli1* and *gli2* genes are downstream targets of sonic hedgehog (*shh*), which is a key gene in early cellular patterning processes. At the 4 to 5 somites stage in zebrafish, *shh* is expressed in the tip of the ventral neural keel, and its domain of expression is mantained in the ventral diencephalon and rostral forebrain up to the end of somitogenesis (24hpf), where the most rostral *shh*-expressing cells are confined to the hypothalamus (**Krauss et al., 1993**). The fact that in the developing zebrafish embryo the olfactory placodes are intimately associated with the anterior pituitary, hypothalamus and CNC precursors in the anterior neural plate, strongly suggest that any of those tissues could be a source of GnRH cells (**Whitlock, 2004**). Additionally, a recent study of the development of the anterior pituitary gland using a transgenic line expressing GFP under the control of proopiomelanocortin gene (POMC) showed a transient association between GFP-expressing cells and olfactory placodes in formation (**Liu et al., 2003; reviewed in Whitlock, 2004**). These data suggest that the hypothalamic GnRH cells do not originate from the OP, but rather in the anterior pituitary placode or other adjacent areas in the anterior neural plate (presumptive anterior hypothalamus).

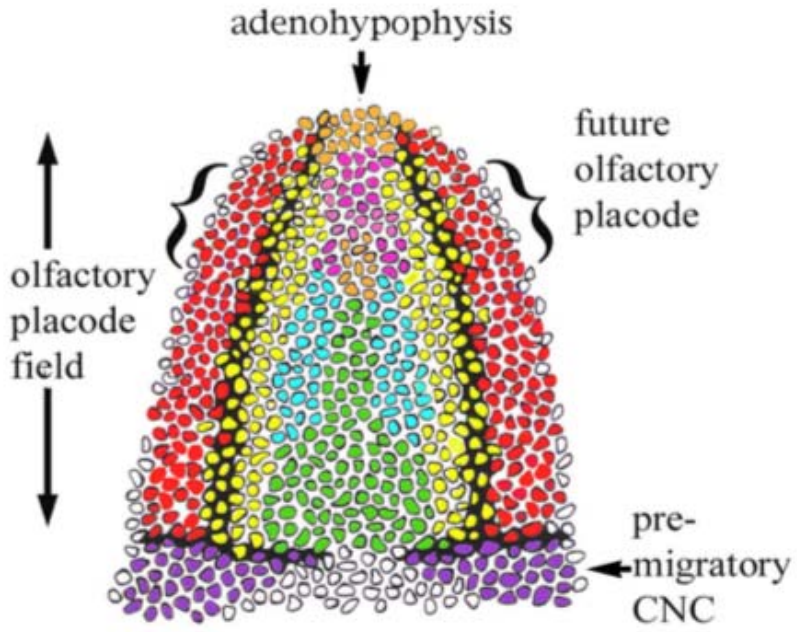


Figure 2. Fate map proposed for the anterior end of the neural plate of zebrafish at 4-6 somites stage (based on comparison with chick). Diagram of a dorsal view of anterior neural plate of the zebrafish, anterior to the top of the page. Regions are color coded to the fate map of chick (**Le Douarin & Kalchein, 1999**). The OP precursors (red) flank the region that give rise to the anterior pituitary placode (orange) (**Whitlock & Westerfield, 2000**). Posteriorly, the OP precursors share a border with the CNC (purple). The hypothalamic precursors (magenta) lie along the midline meeting the adenohypophyseal and OP precursors at the anterior limit. From **Whitlock, 2005b**.

1.2 Hypothalamic GnRH deficiency.

In mammals, the release of hypothalamic GnRH into the median eminence is essential for functioning of the hypothalamic-pituitary-gonad (HPG) axis. This peptide activates the GnRH receptor 1 (GnRHR1) on the anterior pituitary, stimulating expression, synthesis and secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (**Sisk & Foster, 2004**; **de Roux, 2005**). In turn, both LH and FSH stimulate gametogenesis and steroid production in the gonads (**from Cadman et al. 2007**) (**Figure 3**). In humans, the lack of normal GnRH pulsatile pattern of secretion from the hypothalamus results in patients with gonad

failure who do not enter puberty (**Ebling, 2005**). Kallmann syndrome (KS) is a heterogeneous developmental inherited disorder affecting 1:8,000 males and 1:40,000 females (**Hu et al. 2003**) characterized by hypogonadotrophic hypogonadism (HH) associated with anosmia (**Kallmann et al., 1944**). These shared characteristics, anosmia and lack of pulsatile GnRH (**MacColl et al., 2002**), suggest a developmental link between reproductive axis and the olfactory system. Phenotypes of KS patients vary in severity and defects can be observed in nervous system, midline structures (such as cleft palate), and organogenesis (from **Cariboni and Maggi, 2006**). Thus in addition to GnRH deficiency and anosmia, there is a spectrum of phenotypic defects observed in KS patients.

KS is characterized by three modes of familial inheritance, namely X chromosome linked (KAL1), autosomal dominant (KAL2) and autosomal recessive. The KAL1 defects account for approximately 15–20% of total KS (**Quinton et. al., 2001**), and the gene that underlies this form of KS encodes a secreted glycosylated protein called *anosmin 1*. The protein has an approximate molecular mass of 100kiloDalton (kDa) that it is processed on the cell membrane to yield a 45kDa component of extracellular matrices that binds tightly to cell surfaces (**Rugarli et al.,1996**).

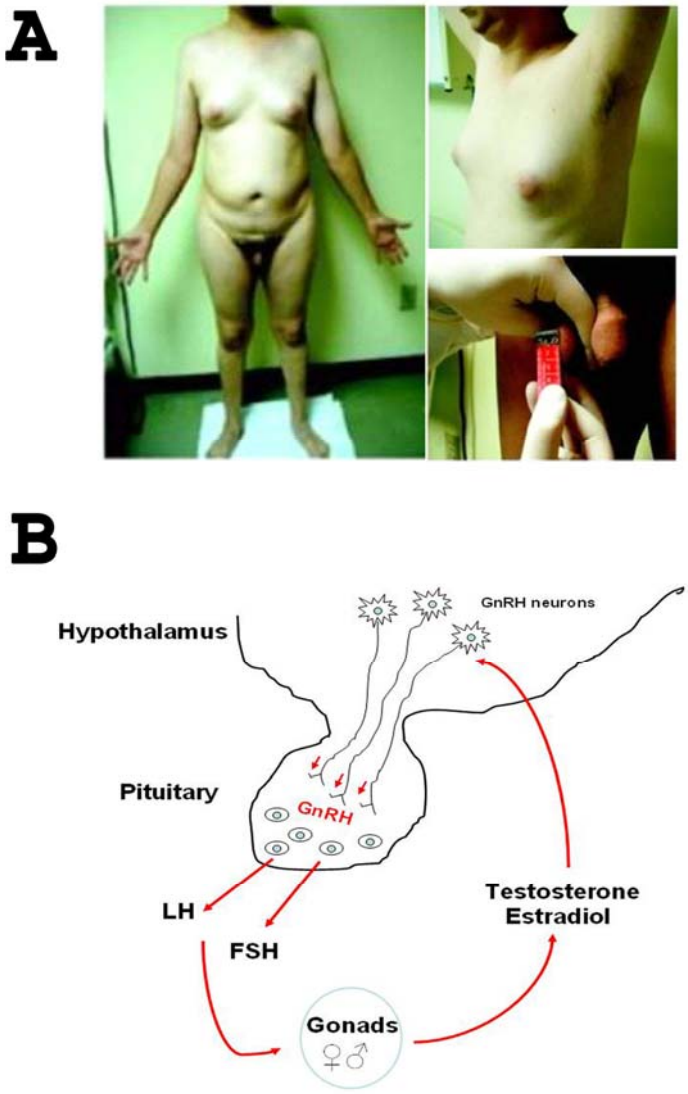


Figure 3. Release of hypophysiotropic GnRH to the anterior pituitary is essential for functioning of the hypothalamic-pituitary-gonad axis. (**A**) Picture of a 33 years old male patient with Kallmann Syndrome. Little body hair, small testes, micropenis, gynecomastia, eunuchoidism and mirror movements are some characterists of the disease (**Meza-Vázquez et al., 2011**). (**B**) Overview of the hypothalamic-pituitary-gonad (HPG) axis, showing hypothalamic GnRH neurons projecting to the pituitary gland. LH and FSH released from the pituitary gland stimulate gametogenesis and steroid production essential for puberty development in the gonads. LH: Luteinizing hormone, FSH: Follicle-stimulating hormone.

Mutations in the KAL2 gene account for 10% of KS phenotypes (**Dodé et al., 2003**) and this gene encodes fibroblast growth factor receptor 1 (*fgfr1*) (**Coumoul &**

Deng, 2003). Nearly 80% of the KS patients, however, do not carry a mutation in KAL1 or KAL2 genes (**Tsai & Gill, 2006**) suggesting that there are other genes underlying KS. A third category of KS arising from mutations in the prokineticin receptor-2 (*prokr2*) and prokineticin-2 (*prok2*) genes cause an autosomal recessive form of KS (**Dodé et al., 2006; Pitteloud et al., 2007; Abreu et al., 2008, Leroy et al., 2008**), referred to as KAL3 (**Dodé & Hardelin, 2010**).

Two homologues of the *anosmin 1* gene have been identified in zebrafish: *kallmann 1a* (*kal1a*) and *kallmann 1b* (*kal1b*) (**Ardouin et al., 2000**), as well as the *fgfr1* gene (**Scholpp et al., 2004**). These genes have a dynamic pattern of expression during early development that includes the olfactory system and hypothalamus among other tissues. A recent study showed that knockdown of *kal1a* and *fgfr1* gene function resulted in the loss of endocrine GnRH cells in addition to disruptions in the olfactory system and presumptive hypothalamic tissue (**Whitlock et al., 2005a; Kim et al. 2006; Kim et al. *unpublished results***). These results suggest that depletion of *kal1a* and *fgfr1* proteins affects normal pattern formation in the anterior neural plate, thus influencing the correct development of the hypothalamus as well as anterior pituitary and the olfactory system (**Whitlock et al. 2006**). In contrast to KAL1 and KAL2, the genes underlying KAL3 (*prokr2* and *prok2*) have not been isolated in zebrafish.

1.3 Prokineticins and GnRH deficiency.

Prokineticins (PROK1-2), are secreted bioactive proteins that possess 10 conserved cysteines, which form 5 disulfide bonds that make this peptide highly

compact (**Li et al., 2001**). In mouse, the mature forms of PROK1 and PROK2 consist of 86 and 81 amino acids respectively having ~40% identity in their primary structure (**Matsumoto et al., 2006**). The N-terminal domain of these proteins contains the hexapeptide AVITGA which is a sequence essential for the biological activity and is highly conserved from fish to humans (**Kaser et al., 2003**). The functions of PROK2 include intestinal contraction, ingestive behavior, hyperalgesia, neuronal survival, angiogenesis, spermatogenesis, hematopoiesis and circadian rhythms (**for review see Zhou, 2006, Negri et al., 2007**). The prokineticin receptors, 1 and 2 (PROKR1 and PROKR2) are G protein-coupled receptors, which show a strong similarity (87% homology, **Matsumoto et al., 2006**) and mediate signal transduction of prokineticins (**Lin et al., 2002; Masuda et. al., 2002; Soga et al., 2002**). In humans, PROKR1 is found in peripheral tissues, whereas PROKR2 is mainly located in the central nervous system (**Kaser et al., 2003**). Analysis has shown that PROK2 binds to both PROKRs with higher affinity than does PROK1, suggesting that PROK2 is the stronger agonist for PROKRs (**Lin et al., 2002; Masuda et al., 2002; Soga et al., 2002**). PROKRs can couple to Gq, Gqi and Gs proteins activating multiple intracellular transduction pathways that finally promote intracellular calcium mobilization (**Lin et al., 2002, Chen et al., 2005**).

In contrast to what is observed in PROKR1 knock-out (KO) mice, loss of receptor function in PROKR2 KO mice results in olfactory bulb (OB) hypoplasia and abnormal layering of the OB as well as severe gonadal atrophy and lack of hypothalamic GnRH cells (**Matsumoto et al., 2006, Prosser et al., 2007**). Furthermore, mRNA levels of FSH and LH were significantly lower in KO animals than wild type controls. Thus, it appears that activation of PROKR2 but not

PROKR1 is critical for morphogenesis of the OB and the correct development of GnRH neurons. Loss of function of the PROK2 ligand resulted in a similar phenotype of reduction in the OB size and abnormal OB cytoarchitecture, suggesting that, like PROKR2, PROK2 plays an important role in OB formation (**Ng et al., 2005**). The PROK2 KO mice have phenotypes that are similar to phenotypic patients with HH; they do not pass through puberty and are infertile, have low serum LH and FSH and have fewer GnRH-expressing cells in the hypothalamus (**Pitteloud et al., 2007**). Unlike the GnRH phenotype, OB malformation was reported in only 50% of the PROK2-/- animals (**Ng et al., 2005**). Thus *prokineticin2* mutations lead to defects in GnRH cell development that is not necessary correlated with OB disfunction (**Pitteloud et. al, 2007**).

These phenotypes are similar to those observed in human patients, where hypogonadism and anosmia can exist separately or together in the same lineage, suggesting that olfactory development may not always be related to the development of GnRH cells (**Carboni & Maggi, 2006**). Furthermore, mutations in *prok2* and *prokr2* genes were found in patients with normosmic idiopathic HH (IHH) suggesting that both *prok2* and *prokr2* play a role in GnRH neuron development in addition to OB formation (**Cole et al., 2008**).

As mouse *prok2* genes are expressed in the olfactory bulb and in the hypothalamus (suprachiasmatic nucleus, medial preoptic area, and nucleus arcuate) and are involved in the development of those KS affected structures, we propose that zebrafish has homologues of mouse *prok2* genes and that these genes are expressed in telencephalic and ventral diencephalic domains of the embryos and the adult brain, as seen in mammals. According with this idea, we

also propose that knockdown of *prok2* genes affect the correct development of the olfactory system and the hypothalamus in zebrafish embryos (**Figure 4**).

Here we report the identification of the *prok2* and *prokr2* genes in the zebrafish. Through gene knockdown techniques we show that disruption of *prok2*, *prokr2a* and *prokr2b* gene function results in abnormal development of the olfactory system and hypothalamic cell types, a phenotype related to the defining clinical anomalies of patients with KS.

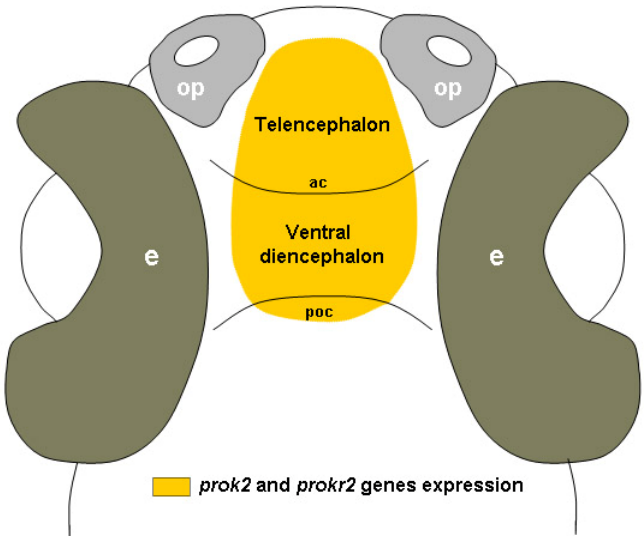


Figure 4. Proposed domains of expression of *prokineticin2* signaling pathway genes. We propose that zebrafish has homologues of mouse *prok2* and *prokr2* genes and that these genes are expressed at least in the telencephalic and ventral diencephalic regions of the embryos (orange). As a result of the proposed domains of expression, knockdown of *prok2* genes should affect the olfactory system (telencephalon) and hypothalamus (ventral diencephalon) development. e: eye, op: olfactory placode, ac: anterior commissure, poc: posterior optic commissure.

2. MATERIALS AND METHODS.

2.1 Animals.

Zebrafish (*Danio rerio*) wild type embryos from “new wild-type” (NWT) and “Cornell” strains (both developed in the Whitlock Laboratory) were reared in a recirculating system (Aquatic Habitats Inc, Apopka, FL) in a 14h light/10h dark photoperiodic cycle at 28.5°C. Embryos were collected on the morning of the day of the experiments and staged according to morphological criteria and hours post fertilization (**Kimmel et al., 1995**). Collected embryos were maintained in embryo media (**Westerfield, 1993**) at 28.5°C. Animal procedures were approved by the University of Valparaiso and NIH animal use and care committees. The olfactory marker protein (OMP:YFP) transgenic line (**Miyasaka et. al., 2005**) was obtained from the Yoshihara Lab and the proopiomelanocortin (POMC:GFP) line (**Liu et al., 2003**) from the Melmed Lab and maintained in our fish facility.

2.2 Cloning of zebrafish *prok2* and *prokr2s* genes.

On column (RNase-Free DNase Set, Qiagen) DNase-treated total RNA was isolated (RNAeasy Kit, Qiagen) from several development stages (0hpf to 5dpf) and adult wild type brain (NWT and Cornell strains) and used as the substrate for reverse transcriptase reaction (Superscript II, Invitrogen) following manufacturer’s instructions. *prok2* (GB #FJ641205)*, prokr2a* (GB #FJ648452) and *prokr2b* (GB #FJ648453) genes were amplified by polymerase chain reaction (PCR) using the primers listed in **Table 1**. These primers were designed with Primer3 online

software (**Rozen & Skaletsky, 2000**) using *Mus musculus* sequences for *prok2* (NM_001037539) and *prokr2* (NM_144944.2) genes as templates for basic local alignment sequence tool (BLAST) (http://www.ensembl.org/Multi/blastview) in the ninth released version of the zebrafish genome (Zv9). cDNA sequence of each pair of primers for each gene (**Table 1**) was cloned into pGEM-T Easy Vector System I (Promega) and confirmed by automated sequencing (Macrogen, Inc.). Sequences of *prok2, prokr2a* and *prokr2b* genes were submmited to Gene Bank and an accesion number for each gene was given (#FJ641205 for *prok2* , #FJ648452 for *prokr2a* and #FJ648453 for *prokr2b*). Online multiple sequence alignment was done using BOXSHADE 3.21 from the European Molecular Biology Network (**http://www.ch.embnet.org/software/BOX_form.html**). Multiple sequence alignment was generated using ClustalW online software and the resulting MSF/GCG format file was used as input in the BOXSHADE platform. The phylogenetic tree was generated using the free online platform **http://www.phylogeny.fr/** (**Dereeper et al., 2008**). The "one click" phylogeny analysis algorithm allows us to reconstruct and analyze the relationship between molecular sequences optimizing each step for our data.

2.3 Morpholinos.

Morpholinos (MOs) were designed and manufactured by Gene Tools Inc. (see www.gene-tools.com for selecting MO antisense oligos details), and then checked for homology with non-target mRNAs in Zv9. MOs were delivered prequantitated, freeze-dried, sterile, salt free and lyophilized solid in an amber glass vial. Each

oligo was solubilized in double distilled water at a final concentration of 1mM (stock solution) for injection into the cytoplasm of one or two cell stage zebrafish embryos (**Nasevicius & Ekker, 2000**). In the solution, solid aggregates were dissociated by heating for 5-10 minutes at 65°C and briefly vortexed. MOs stock solutions were maintained sterile, tightly capped and in the dark at room temperature. For each gene, two different MOs directed against the start site were used. We used mismatch MOs with 5 mis-pairs appropriately distributed along the length of the oligo, to control for gene specificity effects of the MOs. Sequences were as follows: *prok2* MO#1, 5´-TCATGGTCTTCAATGGCTGGTGTC-3´; *prok2* mm#1, 5´-TCATcGTgTTgAATGGCTcGTcTC-3´; *prok2* MO#2, 5´-GAAATGTTGGATGTCATGGTCTTCA-3´; *prok2* mm#2, 5´-GAAATcTTcGATGTgATGcTgTTCA -3´ (GenBank #FJ641205); *prokr2a* MO#1, 5´-GCTGTTTCTGTGAGATGCAAAGTAA-3´; *prokr2a* mm#1 5´-GCTcTTTgTGTGAcATGCtAAcTAA-3´; *prokr2a* MO#2, 5´-ATATTGGCGTCCTGCATGGCTGTTT-3´; *prokr2a* mm#2 5´-ATAaTcGCcTCCTcCATcGCTGTTT-3´ (GenBank #FJ648452); *prokr2b* MO#1, 5´-GTGTTGTTTTCAGTCATCTTGTGTG-3´; *prokr2b* mm#1, 5´-GTcTTcTTTTCAGTgATCTaGTcTG-3´; *prokr2b* MO#2, 5´-CTGTGCTTGTGTTGTTTTCAGTCAT.-3´; *prokr2b* mm#2, 5´-CTcTcCTTcTGTTcTTTTCAcTCAT-3´ (GenBank #FJ648453). All of the MOs used were synthesized with 3´-end modifications for visualization in the embryos post injection. The *prok2* MO#1, *prok2* MO#2 and *prokr2a* MO#2 MOs were tagged with lissamine (red fluorescence) and the *prokr2a* MO#1, *prokr2b* MO#1 and *prokr2b* MO#2 MOs were tagged with fluorescein (green fluorescence). Control mismatch

(mm) were co-injected with rhodamine dextran dye (Invitrogen), at a final concentration of 0.02%. For each MO and mm oligonucleotides, a calibration curve with 1 to 10nl of 1.0, 0.5 and 0.2mM concentration of MO were tested to determine the most effective concentration. For *prok2* MO 1-2nL (0.33mM), *prokr2a* MO 5nL (1.0mM) and *prokr2b* MO 1-2nL (0.2mM) were used for injections. For all control mm MOs we injected 1-2nL, which produced no observable phenotype. For coinjections of *prokr2a* and *prokr2b* MOs equal amounts of the concentrations used above were pre-mixed. Injection pipettes were pulled using thin walled low melting point borosilicate glass tubing with microfilament (OD=1.2 mm, ID=0.94 mm) on a laser-based micropipette puller (P-2000, Sutter Instruments). Parameters used were as follows: heat=350, filament=4, velocity=60, delay=225, pull=150 (Program #32). Further specifications on the use of the puller can be viewed at Sutter web site (www.sutter.com).

For analysis of *otp* (**Eaton and Glasgow, 2007**), *otx2* (**Li et al., 1994**), *oxytocin-like* (**Unger & Glasgow et. al., 2003**), *emx1* (**Kawahara & Dawid, 2002**) expression in the *prok2, prokr2a* and *prokr2b* MO-injected animals, embryos were fixed at 56±1hpf. For *shh* (**Krauss et al., 1993**) expression injected embryos were fixed at 26±1hpf.

2.4 Real-time PCR.

Relative levels of *prok2*, *prokr2a* and *prokr2b* mRNAs were determined by quantitative real-time PCR (qPCR). On column (RNase-Free DNase Set, Qiagen) and on tube (Dnase I, Invitrogen) DNase-treated total RNA was isolated using a

mono-phasic solution of phenol and guanidine isothiocyanate (TRIzol® reagent, Invitrogen) from several development stages (0hpf to 5dpf) and adult wild type brain (NWT and Cornell strains). Total RNA was quantified using both a NanoDrop 3300 spectrophotometer (Thermo Scientific, Inc.) and the Quant-iT RNA Assay Kit (Invitrogen) with a fluorescence microplate reader (Stratagene Mx3000P). Total RNA (1μg) was used as the substrate for reverse transcriptase reaction (Superscript® II, Invitrogen) following manufacturer's instructions and using Oligo dT (Invitrogen) as primers for mRNA isolation. All primer sets used (designed with Primer3 online software and synthesized by Integrated DNA technologies, Inc.), were validated to confirm amplification of a single PCR fragment with a 100% efficiency approximately. qPCR experiments was performed on a Stratagene Mx3000P machine using the double strand DNA dye SYBR Green/ROX qPCR Master Mix (Fermentas) and the conditions were as follows: 10 minutes at 95°C, followed by 40 cycles of 95°C for 15 sec, 57°C for 15 sec and 72°C for 15 sec. Fluorescence intensity was measured during every PCR cycle at selected temperatures. Forward 5´-GTGTTGTGCAGTCAGTCTGTGGAT-3´ and reverse 5´-ACCTTGTGGCTCATTGGATGACAG-3´ primers were used to amplify a 93bp product of *prok2* cDNA. Forward 5´-AATCCACCTACACCTCCAGGAAGA-3´ and reverse 5´-AGTGACGTGACAGACGCTGATTTC-3´ primers were used to amplify a 104bp product of *prokr2a* cDNA. Forward 5´-TTTCAGCCAACAGTGGTGTTGTGG-3´ and reverse 5´-TGCTGAAACACAGAGAGAAATTTGTGG-3´ primers were used to amplify a 84bp product of *prokr2b* cDNA. Quantities of transcripts were normalized according to

levels of *β-actin* transcript using the forward 5´- CGAGCAGGAGATGGGAACC-3´ and reverse 5´-CAACGGAAACGCTCATTGC-3´ oligonucleotides (**McCurley & Callard, 2008**). Relative amount of each sample was determined with a standard curve obtained by amplifying a dilution series of pGEM-T plasmid containing the target fragment sequence. Levels of mRNA accumulation were quantified in two independent experiments and each data point represented the average of triple replicates. For each experiment total RNA was extracted from 100 embryos (for each stage) and 3 adult brains.

2.5 *In situ* hybridization.

Whole-mount *in situ* hybridization was performed as described in Thisse et al. (**Thisse et al., 1993**), using single-stranded RNA probes labeled with digoxigenin-UTP (Roche) and followed with a coloration reaction using NBT/BCIP (Boehringer Mannheim). Agar-embedded (3% in PBS) whole brain were used to obtain sections (70μm) using a vibrating microtome (Pelco vibratome 102). The probes were generated after cloning these cDNAs from zebrafish in pGEM-T easy plasmid. Briefly, the *prok2*, *prokr2a* and *prokr2b* genes were initially cloned using: forward primer, 5´-CTCTGCCTGCTGCTTGTGT-3´; reverse 5´-TGATCTACAGGCACAGAAATGC-3´ (antisense probe was synthesized with SP6 RNA polymerase using NcoI and sense with T7 polymerase using SpeI); forward primer, 5´- ATCACAGGAGTGTGGGTCGT-3´; reverse 5´-TGACGAAGCAGAAGGTGTTG-3´ (antisense probe was synthesized with SP6 RNA polymerase using SphI and sense with T7 polymerase using SpeI); forward

primer, 5´-CTCGCTGGCACGTTACAAG-3´; reverse 5´-GCCGGGTAAAAATCTCGTAG-3´ (antisense probe was synthesized with SP6 RNA polymerase using NcoI and sense with T7 polymerase using NdeI); respectively. Sense RNAs were synthesized and used as negative controls. cDNA was amplified by RT-PCR, the products were cloned into pGEM-T Easy Vector System I (Promega) and the identity of the resulting clones confirmed by sequencing (Macrogen, Inc.).

To make RNA probes for ISH, products cloned into pGEM-T Easy Vector were used for DH5α cells transformation following manufacturer's protocol (Invitrogen). 50μL of transformed bacteria was plated on LB plates with appropriate antibiotic and incubated overnight (O/N) in 37ºC incubator. Transformant colonies were streaked onto new LB plate and incubated O/N in 37ºC incubator. 5mL LB broth in culture tube (with antibiotic) was inoculated with transformant colonies and incubated at 37ºC in shaker O/N. Miniprep was performed following Quiagen miniprep kit protocol. The plasmid was linearized using appropriate enzymes for to 2 hours in a water bath. Gel extraction of the linearized plasmid was performed using Quiagen gel extraction kit protocol. Gel extracted plasmid concentration was estimated by comparing with standard ladder. Sense and antisense digoxigenin (DIG) labeled RNA probe was synthesized using Roche DNA/RNA labeling kit.

The method used for brain or embryo cryostat sections was the following: samples were fixed in 4% paraformaldehyde (PFA; Electron microscopy Sciences) O/N (brains were dissected in 4% PFA with clean forceps) and washed in fix buffer (8 grams sucrose, 150uL 0,2M CaCl2, 0.2M PO4 to 100mL, pH 7.3) 3 times for 5

minutes each. Samples were then tranferred to flexible cryomold (Tissue-Tek) and a drop of clean agar-sucrose solution (1.5% agar, 5% sucrose) at 45°C was added to cover the sample. Clean forceps were used to move the brain or embryos around for proper orientation of the samples. Once solid, the block was removed from the mold and trimmed with a clean razor blade on a hard surface to the desired plane of sectioning. The block was then placed into 30% sucrose O/N for cryoprotection. A drop of OCT compound (Tissue Tek) was added to the cryostat chuck to position the sample and the whole unit was submerged into a container with liquid nitrogen (gradual freezing was necessary to avoid cracking of the block). The block was equilibrated to the cryostat temperature (-25°C) and slices (10 to 40µm thick) were obtained and placed in Poly-L-Lysine coated slides (Electron Microscopy Sciences). Slices were washed in clean 1X PBS to continue with the protocol. Slides were kept in metal humidity chambers and covered for incubations (50mL basal prehybridization solution without tRNA or heparin was added to soak paper towels in the bottom of the humidity chamber). Samples were Prehybridized with hybridization solution (25mL formamide, 12.5mL 20X SSC, 50µL Tween 20, 460µL citric acid pH 6.0, 50µL heparin 50mg/mL, 500µL tRNA 50mg/mL and up to 50mL of DEPC water) at 65°C for 30 minutes and *in situ* probes were preincubated for 10 minutes at 65°C in a waterbath. Hybriwell plastic coverslip (HBW75, Grace Bio-Labs) were used following manufacturer instructions to hybridize O/N at 65°C in steel humidity chambers. Unbound probe were removed by several washes at 65°C.(10 minutes in 66% hybridization solution 33% 2X SSC, 10 minutes in 33% hybridization solution 66% 2X SSC, 10 minutes in 100% 2X SSC, 10 minutes in

100% 0.2X SSC and 10 minunes in 100% 0.2X SSC). Sections were equilibrated at RT for incubation with antibody with the following 5 minutes washes: 66% 0.2X SSC 33% PBST, 33% 0.2X SSC 66% PBST, 100% PBST. Slices were preincubated in 500μL of blocking solution (2mg/mL BSA, 2% sheep serum, 0.1% tween 20) for 30 minutes at RT and then incubated O/N at 4°C with alkaline phosphatase-conjugated antidigoxygenin antibody (Roche; 1:5000). After several washes, 500μL of coloration reaction (1M Tris HCl pH 9.5, 1M $MgCl_2$, 5M NaCl, 0.1% tween 20) buffer was added in the dark until reaction was complete.

The protocol used for whole mount *in situ* of zebrafish adult brain was similar to the whole mount zebrafish embryos ISH (**Thisse et al., 1993**) with the following modifications: brains were incubated in proteinase K (20ug/mL) for 45 minutes at and then fixed in clean 4% PFA for 20 minutes. After hybridization brains were placed in 3% agar 1X PBS solution at 45°C, oriented as desired and cooled to RT. The block was then placed in the vibratome chamber with a drop of cyanoacrylate glue and covered with 1X PBS. The same protocol used for ISH vibratome floating sections was carried out in the next steps, with the difference that RNAse free system was not requiered.

For brain vibratome (Pelco 102 vibratome sectioning system) floating sections the protocol used was as follows: O/N fixed heads in 4% PFA were dissected to remove the brain. After several washes in 1X PBS, samples were placed in methanol for long term storage. Samples were then washed, transferred to a flexible mold (Tissue-Tek) and covered with 1X PBS 3% agar solution. 2-3mm of agar around the tissue and 3-4mm of agar below the tissue where leaved to

mount the block in the vibratome chamber. The block was placed in the chamber with a drop of cyanoacrylate glue below the tissue and covered with clean 1X PBS. Slices (70µm thick) were cut and placed in a sterile 24 well plate (with 6 or 7 slices per well) in 1X PBS. Floating sections were incubated in proteinase K (10ug/mL) for 10 minutes at RT, washed with PBST and fixed with 4% PFA for 15 minutes. Samples were prehybridized with hybridization solution at 65°C for 1 hour and incubated with appropriate dilution of probe O/N at 65°C in humidity chamber. Washes, antibody incubation and coloration reaction was performed as described above for cryostat sections.

After coloration reaction, samples from all the methods used were washed for 5 minutes in distilled water, two times for 5 minutes in hiTE buffer (10mM Tris, 2mM EDTA pH 8.0, 0.1% Tween 20), 5 minutes in water and 1X PBST for 15 minutes (or O/N). Slices were then post fixed in 4% PFA 1X PBS for 30 minutes, dehydrated in glycerol series and mounted.

For dehydration and mounting, samples were dehydrated by rinsing for 5 minutes each in the following ethanol series: 50% - 75% - 85% - 95% ethanol and two times in 100% ethanol. The tissue was then washed two times for 2 minutes in xylene, mounted in distyrene plasticizer xylene medium (DPX, Electron Microscopy Science) and a coverslip was added on a flat surface. For mounting in glycerol, samples were rinsed in a glycerol series (25% - 50% - 70% - 90% in distilled water) for 5 minutes each and a drop of 100% glycerol in the slide was added to mount the sample.

For sequences larger than 300bp, we hydrolyzed the probe to a size of 150-200bp. To hydrolyze probes, we incubated the probe at 60-65°C for a time calculated using the following base hydrolysis formula (**Cox et al., 1984**): time in minutes= (LI-LF)/0.11(LI)(LF), where LI=length of insert in kb and LF=desired final length. The hydrolysis reaction was stopped by adding 10ul of 10% glacial acetic acid, 6ul 3M NaOAc, 2ul glycogen (20 ug/ul) and 440ul 100% ethanol to precipitate RNA at -20°C.

2.6 Inmunohistochemistry.

GnRH immunohistochemistry was performed as reported (**Whitlock et al., 2005a**) using the LRH13 monoclonal antibody that recognizes forms of GnRH containing serine at position 4 and tyrosine at position 5 of the GnRH decapeptide (**Park and Wakabayashi, 1986**). This antibody recognizes both the terminal nerve GnRH (TN-GnRH) and H-GnRH cell populations. Antibody labeling was visualized with goat anti-mouse secondary (1:200; Covance) followed by mouse peroxidase anti-peroxidase (1:500; Covance) and DAB (diaminobenzidine, Sigma; 0.05%) coloration reaction. We used a rabbit polyclonal PROK2 antibody (1:200 to 1:2000) raised against aminoacids 35-85 mapping within an internal region of human PROK2 (H-51 sc67176, Santa Cruz Biotechnology, Inc), to determine zebrafish PROK2 protein expression in embryos and adult brain, but we were unable to see any expression with this antibody using a variety of protocols.

2.7 Olfactory bulb and epithelium size measurements.

Dechorionated embryos at 36±1hpf were anesthetized with tricaine (**Westerfield, 1993**) and mounted ventral side up in 3% methylcellulose. Photos were taken and the area covered by the olfactory bulb and olfactory organ was measured using ImageJ (**Rasband, 1997-2009**). Each olfactory organ was analyzed independently (two per embryo). The data collected was normalized by the distance between the centers of the lens of the eyes for each embryo to standardize the measurements by the head size. Unpaired t test was performed using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com.

2.8 Statistical analysis.

Quantitative results were analyzed using GraphPad Prism 5 software. Student *t* test was used for OB and olfactory organ measurements and Wilcoxon matched-pairs rank sum test was used for cell counts. $p<0.001$ was considered statistically significant.

3. RESULTS.

3. 1 Sequence analysis.

To identify zebrafish genes homologous to mouse *prokineticin receptor2* (*prokr2*) and *prokineticin2* (*prok2*) genes, we BLASTed (using BLASTN in Ensembl Genome Browser, **http://www.ensembl.org/index.html**) the nucleotide sequence of *prokr2* (NM_144944.2, GI:31542916) and *prok2* (NM_001037539, GI:82830394) mRNAs against the zebrafish genome (Zv9 version). We found two homologoues of the mouse *prokr2* gene with 69% (*prokr2a*), and 62% (*prokr2b*) at the aminoacid level (**Figure 5A, B**) and one homologue of the *prok2* gene with 65% (*prok2*) identity in the amino acid sequence (**Figure 5C**). Transcripts homologous to the *prokineticin2* receptor named *prokr2a* (XM_001338648.2, GI:189526591) and *prokr2b* (XM_001338502.2, GI:189515973), are located on disctinct chromosomes (13 and 1, respectively). Both genes are comprised of 2 exons and 1 intron as in mammals. Amino acid sequence analysis of these proteins suggests that both are seven transmembrane G protein-coupled receptors (GPCR), similar to what has been reported for mouse PROKR2. When searching with both amino acid sequences for similar receptors in the GPCR database (**http://mrs.cmbi.ru.nl/mrs-3/blast.do**), we found a high degree of identity with prokineticin receptors of various different mammals (not shown). This *in silico* analysis strongly suggests that these genes are GPCRs and further confirms their identity as the homologues of PROKR2 in mammals. Protein sequences of the zebrafish *prokr2* receptors is remarkably conserved between the two receptors, displaying approximately 76% identity at the

aminoacid level. The major sequence differences between zebrafish and mouse PROKR2 receptors are in the first extracellular domain and in the fourth cytoplasmic domain (**Figure 5A, B;** ED1, ID4), while the seven transmembrane domains displayed higher degree of identity (**Figure 5A, B;** TM1-7).

The analysis of the mRNA homologous to the mouse *prokineticin2* (XM_001342467.2, GI:189520392) revealed that this gene is located in chromosome 6 of the zebrafish genome and has 3 exons and 2 introns as in mouse. The amino acid structure shows two important features found in mouse PROK2: (1) the N-terminal AVITGA domain (**Figure 5C**, red box) essential for the activity of the peptide (**Kaser et. al., 2003**) and (2) the presence of 10 conserved cysteines (**Figure 5C,** red arrowheads) with identical spacing important for the folding of this highly compact secreted molecule (**Li et. al., 2001**). Additionally, our analysis revealed that zebrafish *prok2* gene is syntenic with the mouse *prok2* gene showing common neighbors within the region flanking the *prok2* genes (**Figure 6A, B,** red arrowheads). Both mouse and zebrafish *prok2* genes are located in chromosome 6. Taken together, this results strongly suggest that this gene is the homologue of the mouse *prokineticin2*.

The complete cDNA sequences for zebrafish *prok2*, *prokr2a* and *prokr2b* have been submitted to GenBank (accession numbers FJ641205, FJ648452 and FJ648453, respectively). Primers used to clone zebrafish *prok2*, *prokr2a* and *prokr2b* genes are listed in **Table 1**. All of the products amplified were automatically sequenced (Macrogen, Inc.). A phylogenetic tree constructed from the available sequences for PROK2, PROKR1 and PROKR2 from a wide range of species is shown in **Figure 7**.

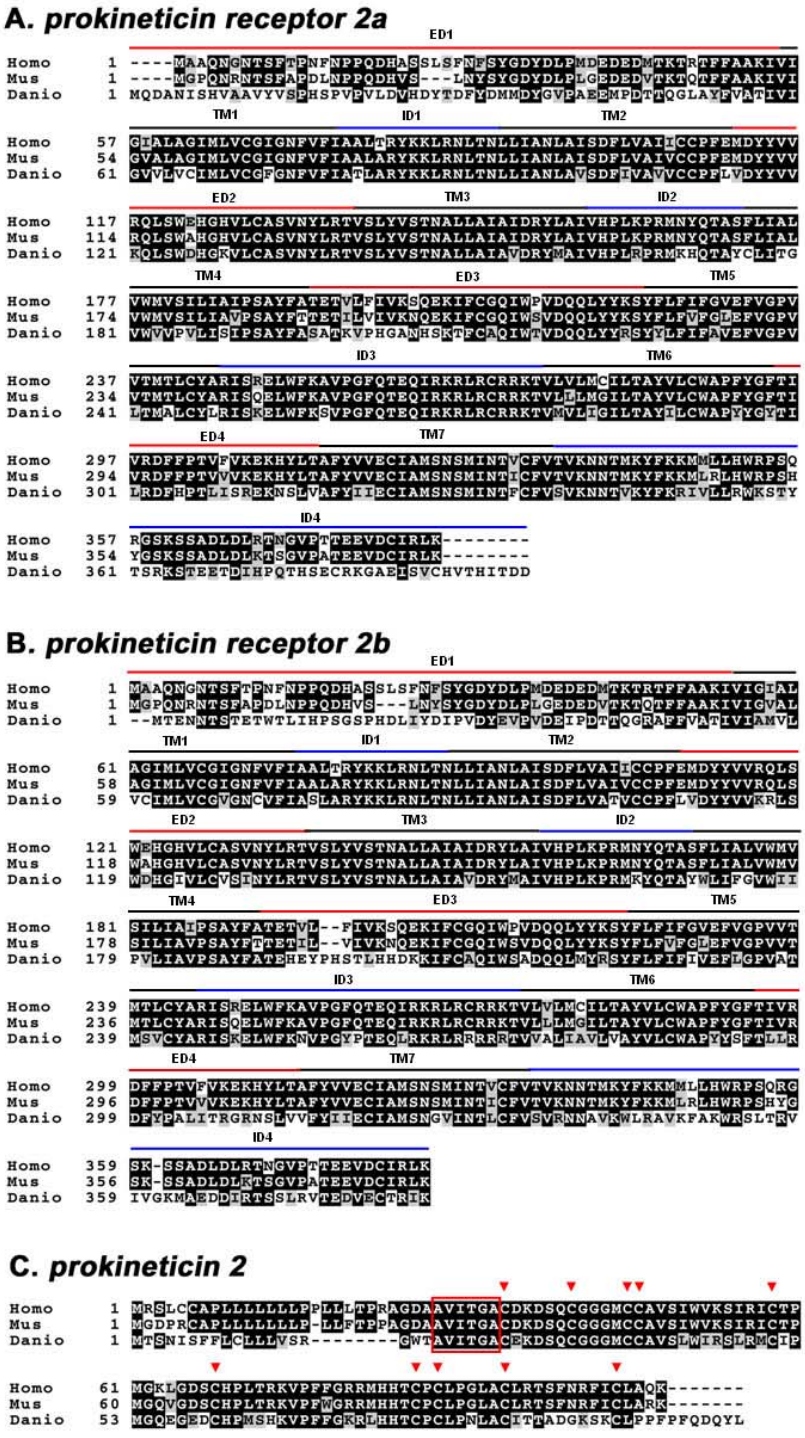


Figure 5. Comparison between amino acid sequences of PROK2 and PROKR2 from different species. Boxshade alignment between amino acid sequences of PROK2 (**A**) PROKR2A (**B**) and PROKR2B (**C**) from zebrafish (*Danio*) with human

(*Homo*) and mouse (*Mus*) proteins. (**A**, **B**) Extracellular domains (ED) are highlighted in red, intracellular domains (ID) in blue and transmembrane segments (TM) in black. (**C**) The AVITGA sequence essential for *prok2* activity is highlighted with a red box, and the ten conserved cysteines are marked with red arrowheads. Black and grey boxes indicate perfect matches and conservative or semiconservative substitutions, respectively. Homo: *homo sapiens*, Mus: *Mus musculus*, Danio: *Danio rerio*.

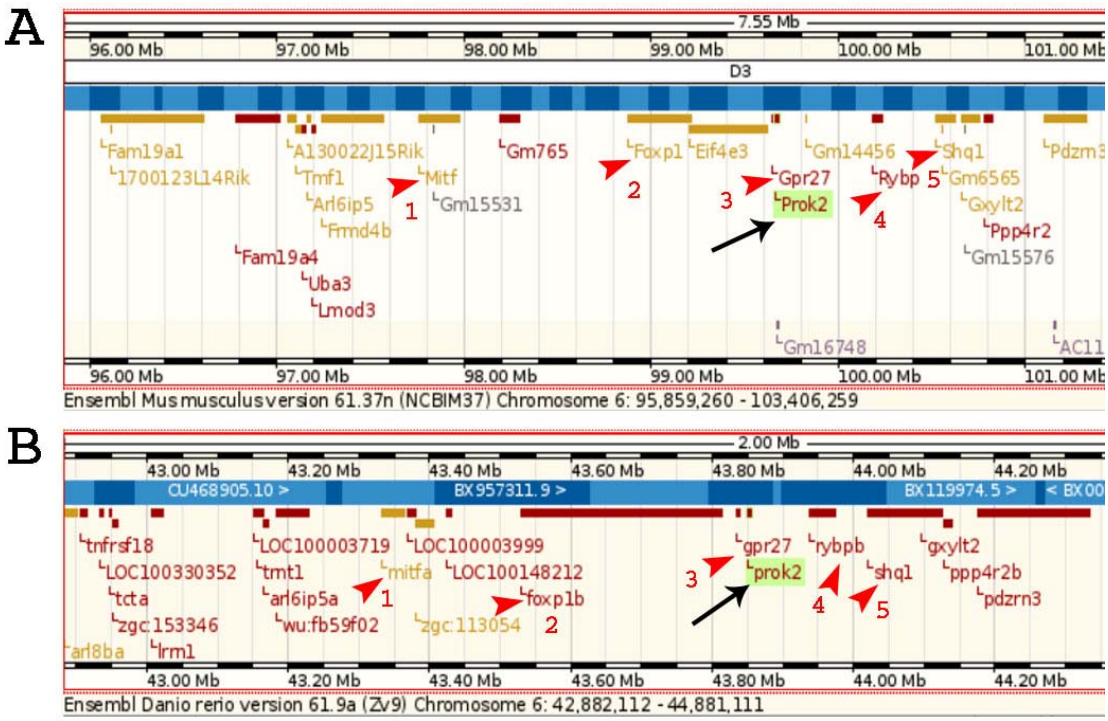
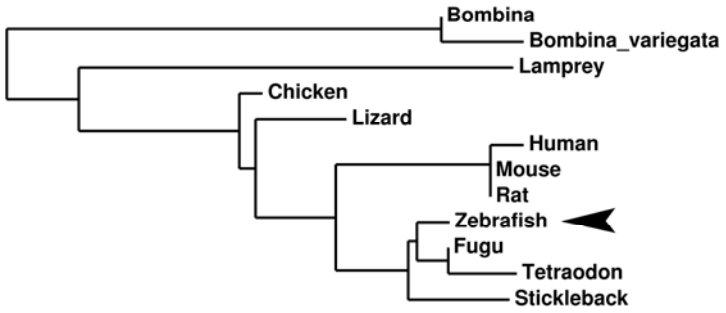


Figure 6. Zebrafish *prok2* gene is syntenic with the mouse *prok2* gene. Bioinformatic analysis show that zebrafish (**A,** black arrow) and mouse (**B,** black arrow) *prok2* genes share common neighbor genes whithin their flanking regions (**A, B,** red arrowheads). Both genes are located in cromosome 6. *In silico* analysis were performed using Ensembl database online software (www.ensembl.org). 1 to 5 in **A, B**: examples of syntenic genes between zebrafish and mouse.

PRIMERS USED FOR EXPRESSION CLONING		
Gene	Sequence (5´-3´)	Name
prok2 (cromosome 6)	gctgcttctcttcacgcttt	prok2cF
	cttctccctcctgacccatt	prok2cR
	ctctgcctgctgcttgtgt	prok2dF
	tgatctacaggcacagaaatgc	prok2dR
	gaggtcatatgatgctaatttatgg	prok2eF
	cgcaaaaaggaatggcatc	prok2eR
	aatgggtcaggagggagaag	prok2fF
	agccgtacacacacatcttca	prok2fR
prokr2a (cromosome 13)	caacaccttctgcttcgtca	prokr2aF
	gcagccgaagtctttaatcg	prokr2aR
	gcatctgccaccaaagtcc	prokr2bF
	acggttttcctccgacatc	prokr2bR
	atcacaggagtgtgggtcgt	prokr2gF
	tgacgaagcagaaggtgttg	prokr2gR
	caacaccttctgcttcgtca	prokr2hF
	tgctattggctgtttttcaa	prokr2hR
	tgatctgtcatcgcacaaga	prokr2iF
	ctgatattggcgtcctgcat	prokr2iR
	gctaaaagtgctaaaccaactgaa	prokr2jF
	ccagagtggcgataaacaca	prokr2jR
	gtggccaccatcgtcatc	prokr2kF
	ctggtccaccgtccagat	prokr2kR
	accgtgatggtgcttattgg	prokr2lF
	tcagtaatatgagtgacgtgaca	prokr2lR
prokr2b (cromosome 1)	gcaacttcggaagcgtctac	prokr2cF
	cctaacgctcacaaagcaca	prokr2cR
	ctggataatcccggttctca	prokr2dF
	ccgggaacattcttaaacca	prokr2dR
	cggcttaaaaggcagtgtgt	prokr2mF
	ggagacacaaagcacaatcc	prokr2mR
	ctcgctggcacgttacaag	prokr2nF
	gccgggtaaaaatctcgtag	prokr2nR

Table 1. Sequences of primers used in amplification and sequencing of zebrafish *prokineticin 2* ligand and receptors. All of the primers shown above were generated using the Primer3 free online software (http://frodo.wi.mit.edu/primer3/). cDNA sequence of each gene was verified by sequencing (Macrogen, Inc.). The products amplified cover the entire coding sequence of each gene and have a certain degree of overlap between them. All this information was uploaded to GeneBank.

A. *prokineticin 2*



B. *prokineticin receptor*

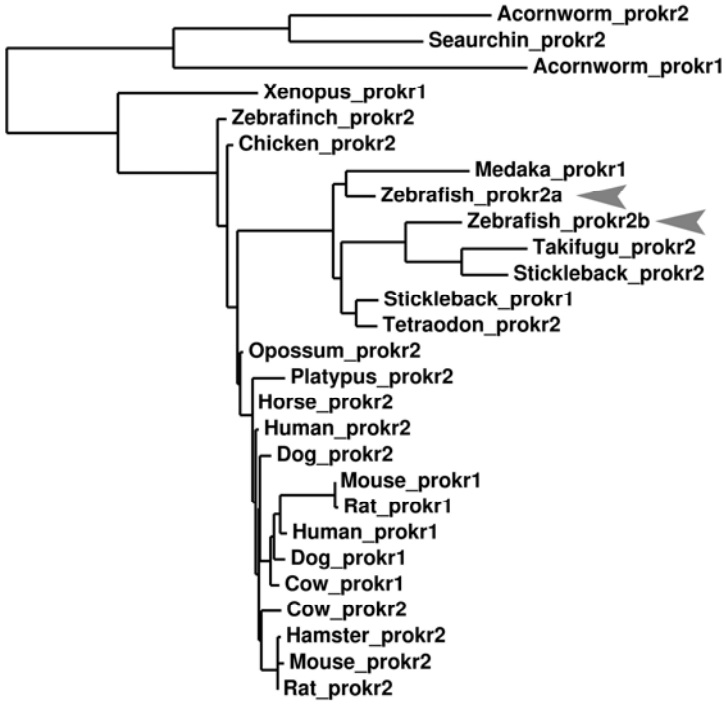


Figure 7. Zebrafish PROK2 ligand and receptors cluster with other fishes. Phylogenetic tree for PROK2 ligand (**A**) and PROK2a and PROK2b receptors (**B**). Zebrafish PROK2 ligand is indicated with a black arrowhead in **A**, and both receptors with gray arroheads in **B**. Note that the phylogenetic distances (support values) between each branch are not shown.

3.2 Isolation and Temporal expression of homologous sequences to *prok2* and *prokr2* genes.

3.2.1 Embryo and adult brain expression by RT-PCR.

In order to examine the temporal expression of *prokr2a*, *prokr2b* and *prok2* genes by RT-PCR we performed DNAse-treated total RNA extraction from several stages (0hpf to 5dpf) for use as substrate for reverse transcriptase reaction. The expression of these cDNAs was examined by PCR using specific set of primers (see methods). We found that the *prok2* gene is first detected at 24hpf, maintaining its level of expression until 5dpf (**Figure 8A**) and that the *prokr2a* and *prokr2b* genes start their expression at least at 24hpf and 12hpf, respectively (**Figure 8A**). To perform region specific microdissections, we used a proopiomelanocortin driven GFP line (POMC:GFP, **Figure 8C**), which is expressed in the anterior pituitary as a marker for the ventral diencephalic region and an olfactory marker protein driven venus (OMP:venus, **Figure 8C**) which is expressed in olfactory sensory neurons as a marker for the anterior telencephalon region. We used the microdissections to determine whether the *prok2*, *prokr2a* and *prokr2b* genes are expressed in specific regions of the brain at 72±1hpf. *prok2*, *prokr2a* and *prokr2b* genes are expressed in the anterior telencephalon (**Figure 8B,** at) and ventral diencephalon (**Figure 8B,** vd) at 72±1hpf and the expression of these genes is reduced or absent in the trunk and tail of the embryos (**Figure 8B,** tt). The *prok2* ligand is expressed in the adult brain telencephalon and diencephalon, but at lower levels in the telencephalon (**Figure 9A**). *prokr2a* and *prokr2b* genes are expressed at similar levels in both

brain tissues (**Figure 9A**). Semiquantitative PCR analysis from dissections of adult zebrafish brain shows that *prok2* transcript is more highly represented in the hypothalamus (**Figure 9B,** hy) than in other brain structures (**Figure 9B**; wh, tl, tc).

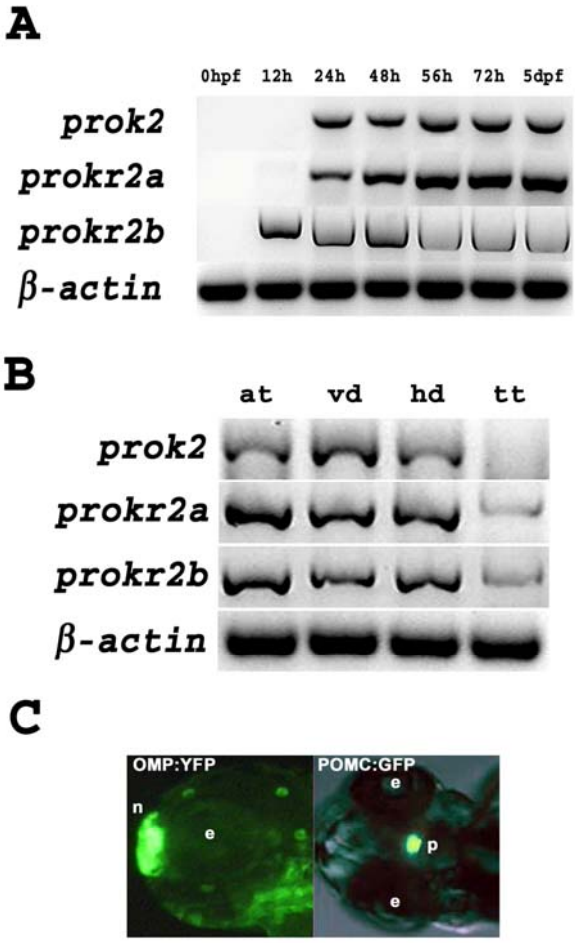


Figure 8. *prok2*, *prokr2a* and *prokr2b* genes are expressed in zebrafish embryos. (**A**) RT-PCR analysis shows that *prok2, prokr2a* and *prokr2b* genes are expressed in zebrafish embryos at different developmental stages. (**B**) *prok2, prokr2a* and *prokr2b* genes are expressed in the anterior telencephalon (at) and ventral diencephalon (vd) in 72hpf zebrafish embryos. at: anterior telencephalon, vd: ventral diencephalon, hd: head, tt: trunk and tail. (**C**) OMP:YFP (lateral view, anterior to the left) and POMC:GFP (ventral view, anterior to the left) transgenic lines were used as markers of olfactory system and ventral diencephalon, respectively. n: nose, e: eye, p: anterior pituitary. All experiments were carried out at least 3 times with 100 embryos for each developmental stage in (**A**) and 50 embryos in (**B**).

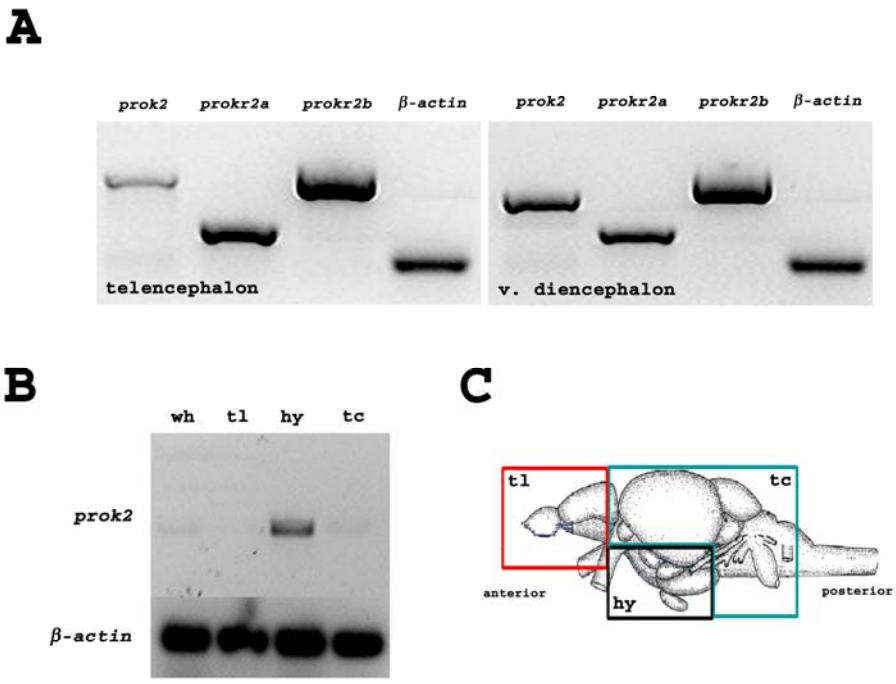


Figure 9. *prok2*, *prokr2a* and *prokr2b* genes are expressed in specific regions of the zebrafish adult brain. (**A**) *prok2, prokr2a* and *prokr2b* genes are expressed in the telencephalon and ventral diencephalon from adult brain. (**B**) Semiquantitative PCR analysis from microdissections of adult zebrafish brain shows that *prokineticin 2* gene is more highly expressed in the ventral diencephalon than in other brain structures. wh: Whole brain, tl: Telencephalon, hy: Hypothalamus, tc: Tectum/cerebellum. (**C**) Diagram modified from **Wullimann et al., 1996** depicting different regions of the brain used in (**B**). 40 PCR cycles in (**A**) and 27 cycles in (**B**) were used for the analysis. All experiments were carried out at least 3 times with 5 brains for (**A**) and (**B**).

3.2.2 *in situ* hybridization expression.

We performed RNA *in situ* hybridization (ISH) using digoxigenin labeled antisense zebrafish *prok2*, *prokr2a* and *prokr2b* as probes. We were unable to observe any signal for the three genes from 24hpf to 5dpf (not shown) embryos using several approaches: 1. we performed wholemount and cryostate sections ISH; 2. at least two probes of different length were used; 3. probes were hydrolized; 4. we changed

the stringency of the protocols (by changing hybridization temperature and formamide percentage/SSC concentration in the post hybridization washes) and 5. we titrate proteinase K, probes and antibody incubation times. In adult brain sections, ISH revealed that the *prok2* gene is expressed in the adult hypothalamic tissue (**Figure 10**). These *prokineticin2*-expressing cells were found specifically in the ventral zone of the periventricular hypothalamus (Hv, **Figure 10E, H**) along the diencephalic ventricle (**Figure 10D-I**) (**Wullimann et al., 1996**). All cells expressing *prok2* were localized only in two 70μm serial sections (140μm in total). We also used another pair of primers (**Ayari et al., 2010**, see methods) to synthesize a different probe for the same gene in order to contrast our results with the expression profile observed in another investigation (**Ayari et al., 2010**), but the results obtained with that probe were the same (not shown) to ours. Our ISH results are consistent with semiquantitative RT-PCR analysis from dissections of zebrafish adult brain, where the *prok2* transcript is more highly represented in the hypothalamus than in other brain structures (**Figure 9B**). We did not detect *prok2* expression in the olfactory bulbs of the adult zebrafish as has been previously reported in mouse (**Cheng et al., 2006**) and zebrafish (**Ayari et el., 2010**) (not shown). As expected, we found no *prokineticin2*-expressing cells with the negative control sense probe (**Figure 10A, B**). We were unable to observe any signal for *prokr2a* and *prokr2b* genes in adult brain sections using several approaches (see below).

Finally, to detect zebrafish PROK2 protein expression, we used a rabbit polyclonal PROK2 antibody (Santa Cruz Biotechnology, Inc) with a concentration

ranging from 1:200 to 1:2000 in embryos and adult brain, but we were unable to see any expression with this antibody (data not shown).

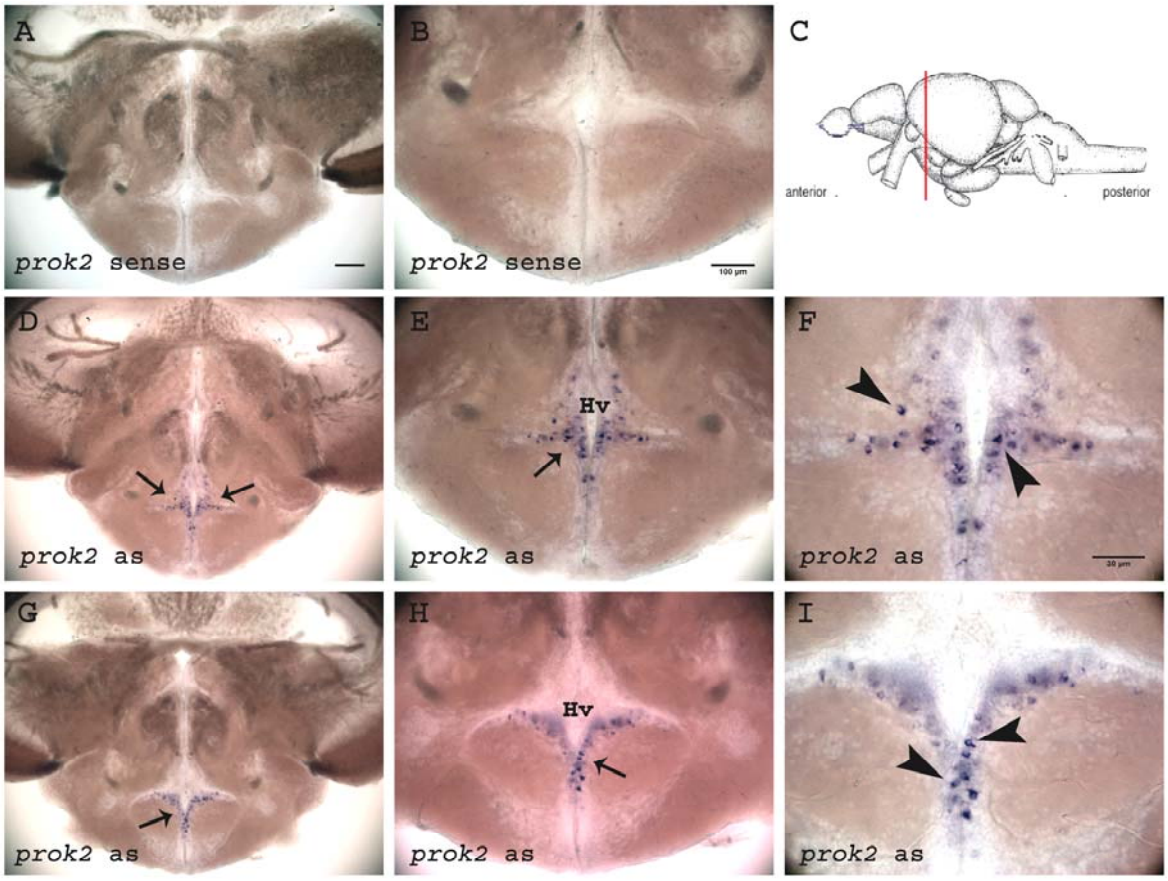


Figure 10. *prokineticin2* is expressed in the hypothalamus of the zebrafish adult brain. Expression of *prokineticin2* in the zebrafish adult hypothalamus was assessed by ISH in brain dissections. Control sense (**A,B**) and antisense (**D-I**) preparations were hybridized with specific DIG-labeled probes before sectioning in a microvibratome. (**D**) picture of a 70µm section with their 20X and 40X magnification (**E** and **F**, respectively) showing *prokineticin2*-expressing cells in the ventral zone of the periventricular hypothalamus (black arrows in **D**, **E**, and black arrowheads in **F**). (**G**) Posterior adjacent 70µm section of (**D**) with their 20X and 40X magnification (**H** and **I**, respectively) showing *prokineticin2*-expressing cells in the Hv (black arrows in **G**, **H**, and black arrowheads in **I**). (**C**) Diagram of a lateral view of adult zebrafish brain illustrating the transverse section (red line) where *prok2* is expressed (modified from **Wullimann et al., 1996**). All pictures were taken with dorsal side to the top. Hv: ventral zone of the periventricular hypothalamus. Scale bar in **A** = 100µm.

3.2.3 qPCR analysis.

To further analyze the expression of *prok2*, *prokr2a* and *prokr2b* genes, we performed qPCR experiments using whole zebrafish embryos and adult brain microdissections. Using this approach, we found that *prok2* (**Figure 11A**), *prokr2a* (**Figure 11C**) and *prokr2b* (**Figure 11E**) genes have a very low level of expression in early developmental stages (from 12hpf to 56hpf) compared to whole adult brain expression (WAB). In addition, we found that in the adult brain *prok2* transcript (**Figure 11B**) is more highly expressed in the ventral diencephalon compared to WAB. Consistent with this result, we were able to detect *prok2* expression by ISH only in the ventral diencephalon (**Figure 10D-I**). *prokr2a* has a level of expression not different from WAB in the regions of the brain used (**Figure 11D**) and *prokr2b* has a 2-fold increase in the ventral diencephalon compared to WAB (**Figure 11F**), although we could not see any expression for these two genes in the adult brain using digoxigenin-labeled antisense probes (not shown).

Taken together, these data demonstrate that homologues of the mouse *prokineticin2* signaling pathway genes (*prok2*, *prokr2a* and *prokr2b*) are present in the zebrafish genome (Zv9) and that these genes are expressed in early developmental stages and in adult brain tissue

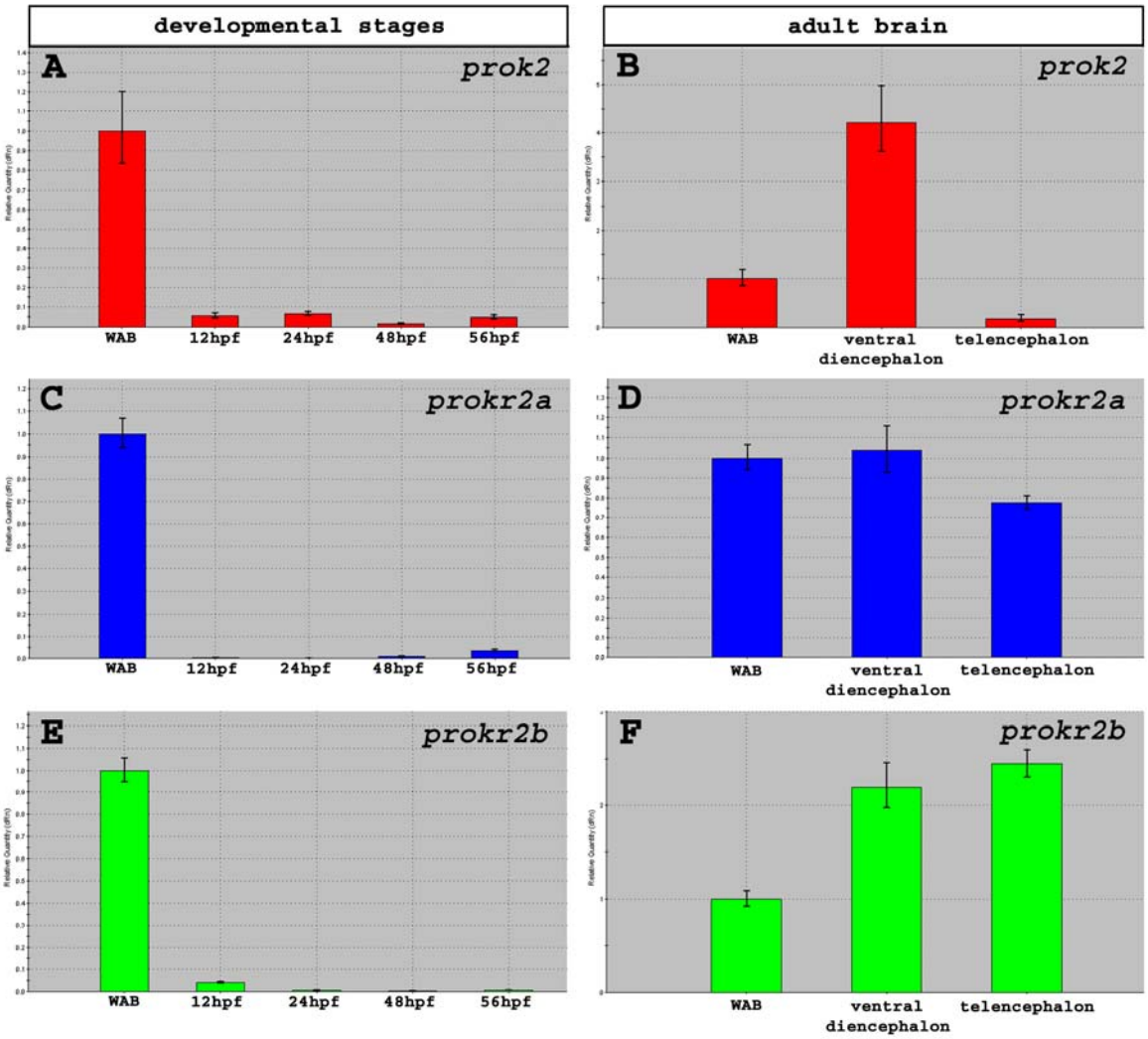


Figure 11. Gene expression assessed by qPCR.
Relative levels of *prok2*, *prokr2a* and *prokr2b* mRNA were determined by qPCR using cDNA from 12, 24, 48 and 56hpf whole embryos (**A, C, E**) and adult brain dissections (**B, D, F**) as substrate. Quantities of transcripts were normalized according to levels of *β-actin* transcript. Levels of mRNA accumulation were quantified in two independent experiments and each data point represented the average of triple replicates. Zebrafish adult brain dissections isolated the regions shown in **Figure 9C**. WAB: whole adult brain expression. hpf: hours post fertilization.

3.3 Knockdown of *prok2*, *prokr2a* and *prokr2b* zebrafish genes.

3.3.1 Morphant phenotypes.

We used specific MOs (designed and manufactured by Gene Tools Inc., and then checked for homology with non-target mRNAs) to knockdown the function of *prok2*, *prokr2a* and *prokr2b* genes. Embryos injected with antisense MOs against *prok2*, *prokr2a* and *prokr2b* genes were similar in appearance at 48±2 hpf (**Figure 12**). The resulting morphant embryos had small head and eyes and the tail was curled ventrally (**Figure 12B-D**). *prok2* morphants (**Figure 12B**) had a small body size and the tail was flexed down (**Figure 12B**). *prokr2a* morphants had a ventrally curved tail (**Figure 12C**), while *prokr2b* morphants had both a flexed trunk and tail (**Figure 12D**). All specificity control mm MOs produced no observable phenotype (**Figure 12A**). We used a different second non-overlapping AUG MO (see methods) against the same mRNAs to block translation of *prok2, prokr2a* and *prokr2b* genes, and the phenotypes produced were very similar to those observed in **Figure 12B-D**.

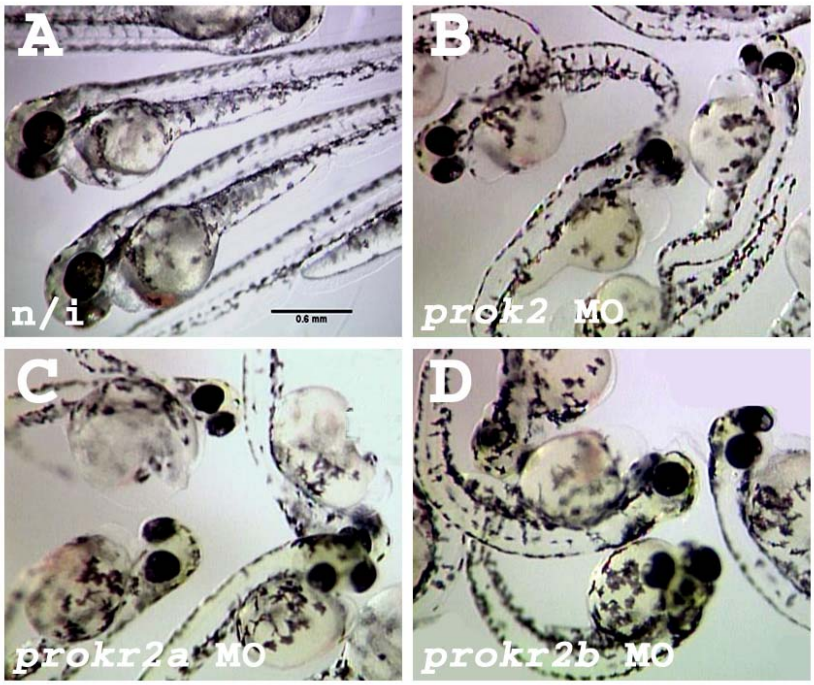


Figure 12. Knockdown of *prok2*, *prokr2a* and *prokr2b* genes in zebrafish embryos produces morphological phenotypes. Morphological defects in embryos resulting from the knockdown of *prok2* (**B**), *prokr2a* (**C**) and *prokr2b* (**D**) genes with MOs at 48±2hpf. All of the resulting morphants were curled ventrally and had small head and body size compared to the control (**A**). n/i: not injected embryos.

3.3.2 Knockdown of *prok2*, *prokr2a* and *prokr2b* genes affects the olfactory system.

Because previous studies using PROK2 KO mouse (**Ng et al., 2005**) have shown a reduction in OB size and abnormal OB cytoarchitecture, we used MOs to determine whether *prok2*, *prokr2a* and *prokr2b* genes have a role in the development of the olfactory system. Thus we measured the size (using ImageJ, see methods) of the olfactory placode (**Figure 13A, B**; red outline) and the olfactory bulb (**Figure 13A, B**; white outline) in uninjected, control mm, and MO-injected animals at 36±1hpf. Our analysis showed that knockdown of *prok2*, *prokr2a* and *prokr2b* genes caused

a significant reduction (p<0,0001) in OB size (**Figure 13C**), but not in olfactory placode (OP) size (**Figure 13D**).

Using the OMP:YFP transgenic line that mark ciliated olfactory sensory neurons (OSNs) and their projections to the OB, we found that the knockdown of *prokineticin2* signaling pathway genes (**Figure 14B-E**) results in abnormal axonal projections of the OSNs to the OB (**Figure 14B-D**, white arrowheads) compared to control mm injected embryos (**Figure 14G-J**) at 72±1hpf. Although OSNs axons entered the OB, their axonal endings are irregular in shape and less defined than those in mm control injected embryos. The axonal projections in the OB of wild type embryos are very characteristic and form three main axon bundles: medial, center, and lateral (**Vitebsky et al., 2005**). In the *prok2*, *prokr2a* and *prokr2b* single morphants the three bundles are visible (L, C and M in **Figure 14G**), but when both receptor MOs are coinjected, the pattern of bundles is affected resulting in nerve morphologies that does not show the characteristic branches (**Figure 14E**, white arrows) observed in the control embryos (**Figure 14G-J**).

The *emx1* gene is expressed in the developing telencephalon (**Morita et al., 1995; Kawahara & Dawid, 2002**) and also has been suggested to guide pioneer axons from the OP to the OB in zebrafish (**Whitlock and Westerfield, 1998**). Thus we used a DIG labelled RNA probe recognizing *emx1* to determine whether telencephalon patterning was disrupted in MO-injected embryos. Using this approach, we found that *emx1* expression is totally absent in the morphants OBs (**Figure 15A-C,** black asterisks**; G-I,** white asterisks) compared with the mm controls (**Figure 15D-F** and **J-L**, white arrowheads) at 36±1hpf (**Figure 15A-F**) and 72±1hpf (**Figure 15G-L**).

Taken together, the data presented above suggest that the *prokineticin2* signaling pathway is important for the normal development of the OB and that the developing OSNs have abnormal axonal projections within the OB as a secondary result of this deficiency.

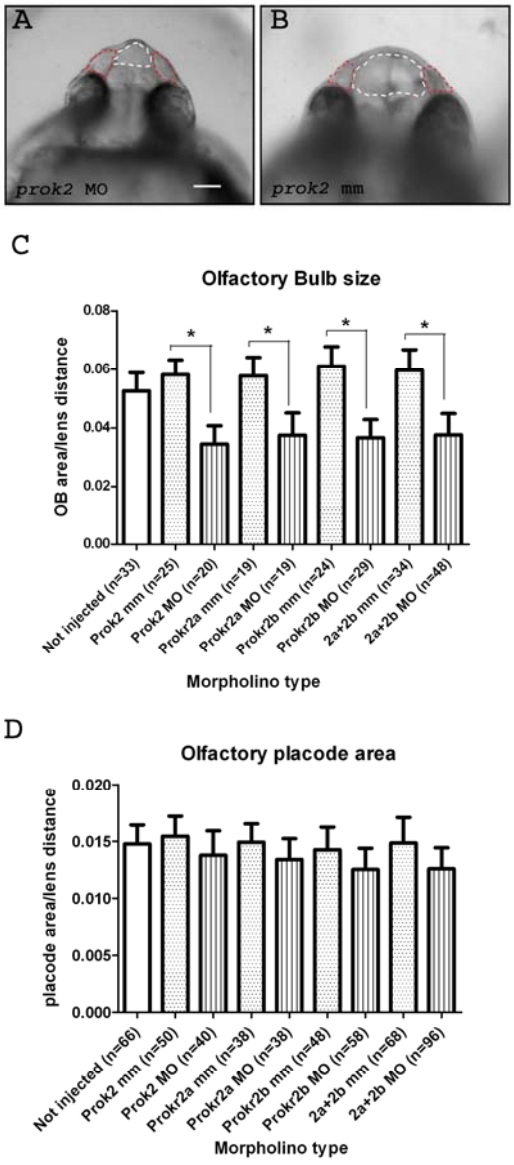


Figure 13. Disruption of *prokineticin2* signaling pathway affects olfactory bulb but not olfactory placode size. Representative ventral view from *prokineticin2* MO-injected (**A**) and mismatch control embryos (**B**) at 36±1hpf, anterior to the top

(OB: white dashed, OP: red dashed line). (**C**) Knockdown of *prok2*, *prokr2a*, *prokr2b* zebrafish genes causes a significant reduction ($p<0.0001$, Student *t* test) in OB size (white outline in **A, B**). (**D**) *prok2*, *prokr2a*, *prokr2b* morphants do not show a reduction in OP size (red outline in **A, B**). (**C**, **D**) White, gray stiple, and gray line bars represent uninjected control, mm control, and MO treated embryos, respectively. Number of animals analyzed are in parenthesis for each treatment in **C**, **D**. All experiments were performed at least 3 times for each MO. mm: mismatch MO injected embryos, MO: morpholino oligo injected embryos. * $p<0.0001$. Scale bar in **A** = 100μm.

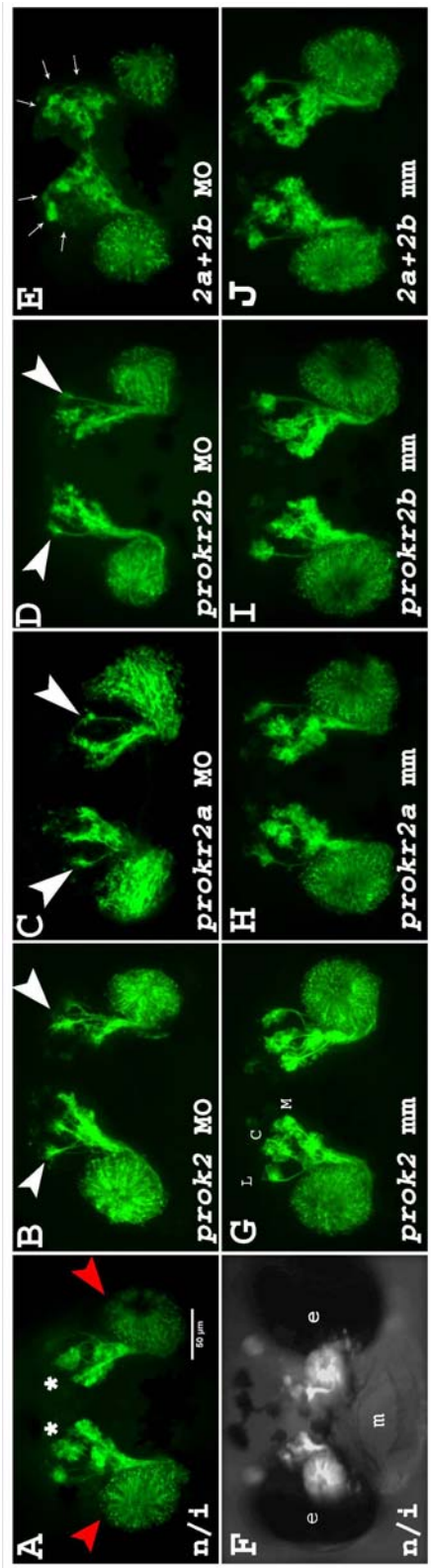


Figure 14. Knockdown of *prok2*, *prokr2a* and *prokr2b* genes in zebrafish embryos affect axonal projections in the OB. Disruption of *prokineticin2* signaling pathway affects the olfactory system. (**A-J**) *prok2*, *prokr2a*, *prokr2b*

knockdown disrupt OSNs axon terminals (white arrowheads in **B-D**) within the OB (white asterisks in **A**) in OMP:YFP transgenic line at 72±1hpf. In addition, the main axon bundles pattern (white arrows in **E**) is affected when both receptor MOs are coinjected. (**A**) uninjected control, (**B-E**) MO injected embryos, (**F**) Frontal view of an uninjected control embryo, dorsal to the top. (**G-J**) control mm MO-injected embryos. All pictures shown are representative preparations and were taken from a rostral view with a 40X objective in an spinning disc microscope (except for **F**), dorsal to the top (**A-J**). All experiments were performed two times for each MO. mm: mismatch control, MO: morpholino oligo, OMP: olfactory marker protein, n/i: not injected, e: eye, m: mouth.

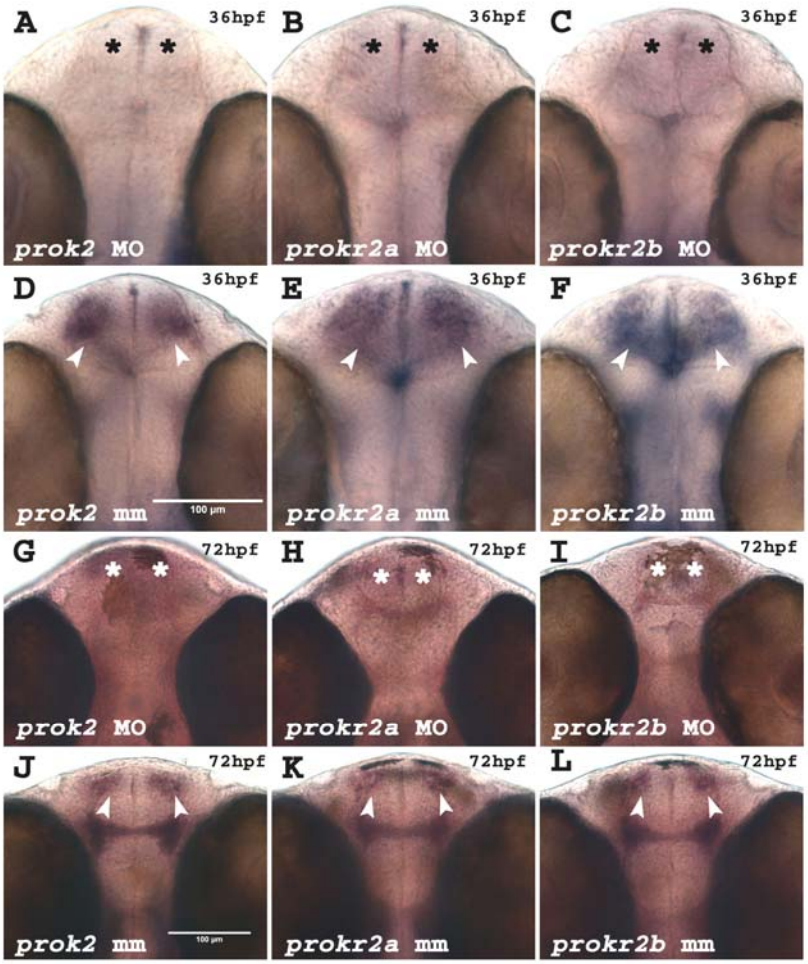


Figure 15. *emx1* expression is absent in *prokineticin2* signaling pathway morphants. *emx1* transcription factor expression is absent in the OBs (black and white asterisks in **A-C** and **G-I**, respectively) of *prok2*, *prokr2a* and *prokr2b* MO-injected embryos at 36±1hpf (**A-F**) and 72±1hpf (**G-L**) compared to the mm control (white arrowheads in **D-E** and **J-L**). (**D-F**, **J-L**) MO mm control. (**A-C**, **G-I**) MO-injected embryos. mm: mismatch control, MO: morpholino oligo, hpf: hours post fertilization. All pictures were taken from a ventral view, anterior to the top.

3.3.3 Disruption of *prokineticin2* signaling pathway affect hypothalamus cell types development.

In order to determine whether loss of function of *prokineticin2* gene affects endocrine GnRH cells from the hypothalamus (H-GnRH), we used the LRH13 antibody (**Park and Wakabayashi, 1986**). We found that *prok2* (**Figure 16B**), *prokr2a* (**Figure 16C**) and *prokr2b* (**Figure 16D**) morphants showed a significant reduction in the number of H-GnRH positive cells at 56±1hpf relative to the normal number of cells observed in mismatch MO-injected controls (**Figure 16F-H**). The *prok2* morphants had an average of 1.1 cells expressing H-GnRH compared with 12.1 cells in the control mismatch (mm) controls (**Figure 18A, C; Table 2**). *prokr2a* and *prokr2b* morphants also showed a reduction in cell number, with an average of 3.0 and 1.7 H-GnRH cells compared with 13.6 and 14.0 in the mm controls, respectively (**Figure 18A, C; Table 2**). In all cases, TN-GnRH cells were not affected by the injection of the MO (**Figure 16B-D**, black arrowheads). The number of H-GnRH and TN-GnRH-expressing cells in mm controls were similar to our previously published results for uninjected wild-type animals (**Kim et al., unpublished results; Gopinath et al., 2004; Whitlock et al., 2005a**).

To further assess the potential role of the *prok2* signaling pathway in hypothalamic development we used a probe recognizing *oxytocin-like* (*OT-like*) gene which is an ortholog to the mammalian peptide gene *oxytocin-neurophysin* and detects bilateral clusters of cells located in the anterior hypothalamus (**Unger & Glasgow et. al., 2003**); (**Figure 17A**). Using this marker, we found that hypothalamic *OT-like*-expressing cells are reduced in number in *prok2*, *prokr2a* and *prokr2b* morphants (**Figure 17B-D**) compared to the control mm MO (**Figure 17F-**

H). The *prok2* morphants had an average of 2.4 cells expressing *OT-like* compared with 19.1 cells in the control mismatch (mm) controls. *prokr2a* and *prokr2b* morphants also showed a reduction in cell number, with an average of 6.5 and 1.8 *OT-like*-expressing cells compared with 25.6 and 24.6 in the mm controls, respectively (**Figure 18B, D; Table 2**). The numbers of *OT-like*-expressing cells in mm controls were similar as we had previously reported for uninjected wild-type animals (**Kim et al., unpublished results**). Using a second AUG MO to disrupt *prok2*, *prokr2a* and *prokr2b* gene expression, we performed the same sets of experiments for recognition of H-GnRH and *OT-like* cell types (**Figure 16 and 17**). We found that the effect of the second MOs were very similar to that observed with the first MOs described above (**Figure 18C, D**).

To determine if the loss of both hypothalamic cells (H-GnRH and *OT-like*) caused by *prok2*, *prokr2a* and *prokr2b* MOs were the result of non-specific effects of the oligonucleotides, we examined the expression of the *orthodenticle 2* (*otx2*) gene in the treated and control embryos at 48±1hpf (**Figure 19**). We found no alterations in the pattern of *otx2* expression at the midbrain hindbrain boundary (MHB) in the morphants (**Figure 19A-C**) when compared with the control mm MO (**Figure 19D-F**, white arrowheads in **Figure 19D**).

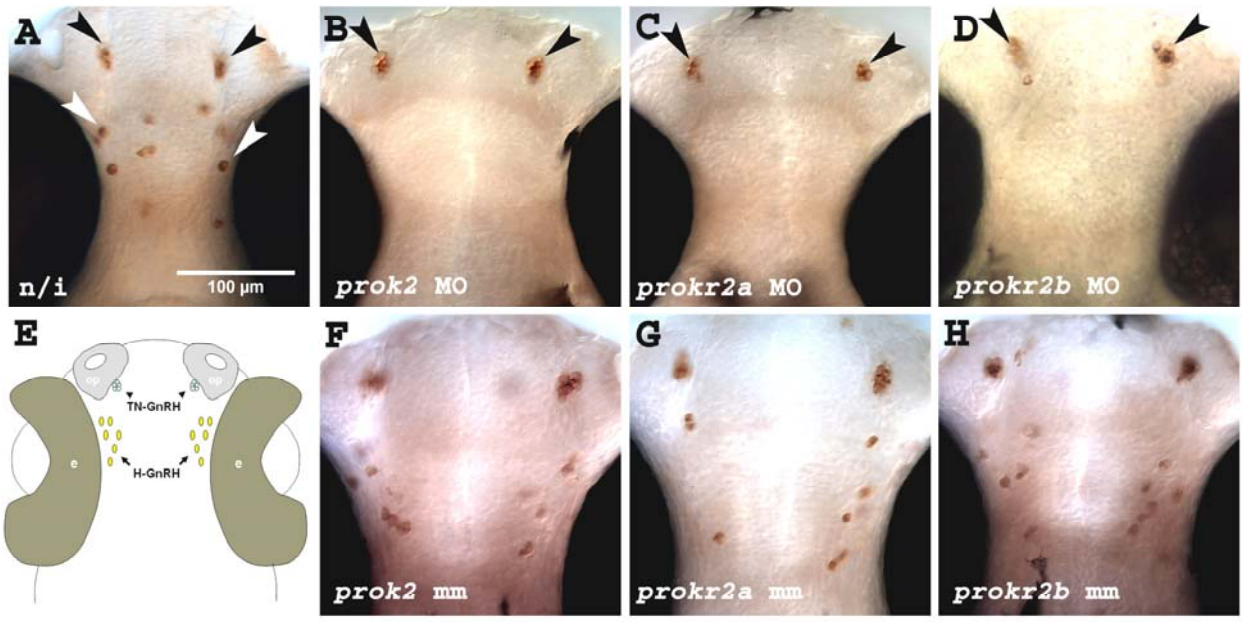


Figure 16. Knockdown of *prok2*, *prokr2a* and *prokr2b* genes cause a reduction in the number of hypothalamic H-GnRH cells in the zebrafish hypothalamus. (**A**) An uninjected embryo showing the location of H-GnRH cells (white arrowheads) and TN-GnRH cells (black arrowheads). (**B-D**) MO injected embryos lack H-GnRH cells compared to embryos injected with mismatch control MOs (**F-H**), but not TN-GnRH cells (black arrowheads). (**E**) Diagram of the location of H-GnRH (black arrows) and TN-GnRH (black arrowheads) cells in a 56hpf zebrafish embryo. op: olfactory placode, e: eye. (**B-D**) Morphants lack H-GnRH cells but TN-GnRH cells are not affected. Ventral views, anterior to the top. mm: mismatch control, MO: morpholino oligo. n/i: not injected.

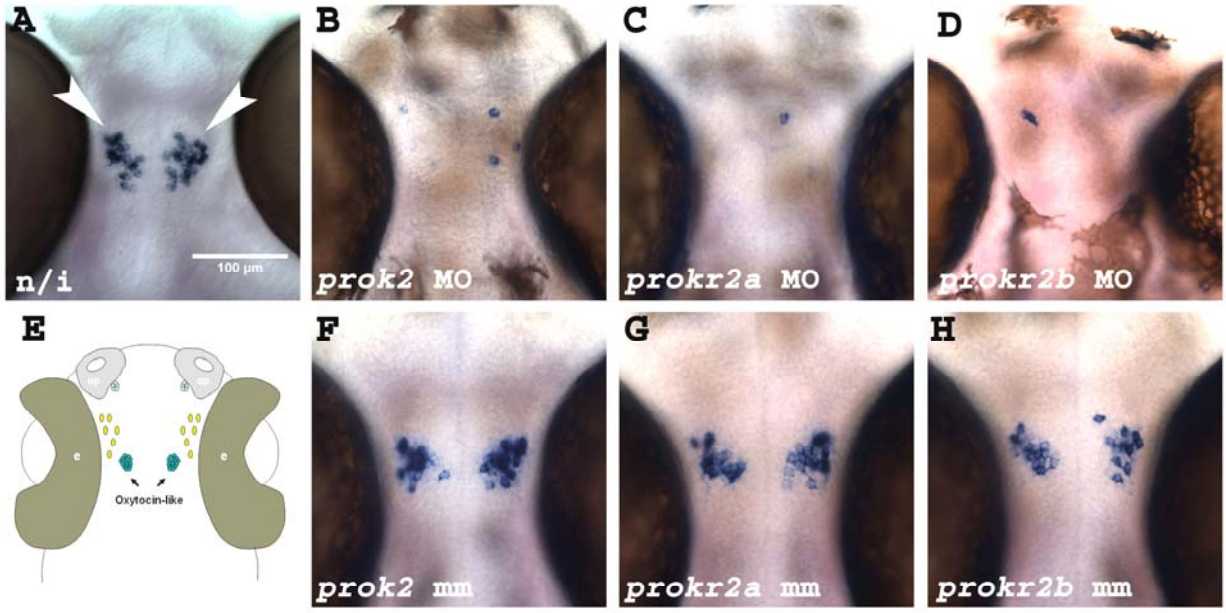


Figure 17. Knockdown of *prok2*, *prokr2a* and *prokr2b* genes cause a reduction in the number of *oxytocin-like* cells in the zebrafish hypothalamus. (**A**) An uninjected embryo showing *OT-like* expressing cells (white arrowheads). MO-injected embryos lack *OT-like* cells (**B-D**) compared to embryos injected with mismatch control MOs (**F-H**). (**E**) Diagram of the location of *OT-like* cells in a zebrafish embryo (black arrows). All pictures were taken from a ventral view, anterior to the top. mm: mismatch control, MO: morpholino oligo. op: olfactory placode, e: eye. n/i: not injected.

Target gene	*n*	min.	median	max.	P-value
Hypothalamic GnRH cells					
prok2 mm	57	2	12	32	
prok2 MO	70	0	0	7	< 0.0001
prokr2a mm	43	5	13	23	
prokr2a MO	67	0	2	12	< 0.0001
prokr2b mm	52	7	14	22	
prokr2b MO	88	0	0	11	< 0.0001
not injected	82	3	13	29	
***Oxytocin-like* cells**					
prok2 mm	40	11	19	28	
prok2 MO	49	0	1	9	< 0.0001
prokr2a mm	46	15	26	35	
prokr2a MO	49	0	4	20	< 0.0001
prokr2b mm	34	17	25	31	
prokr2b MO	38	0	1	6	< 0.0001
not injected	51	19	26	33	

Table 2. Numbers of cells expressing H-GnRH and *OT-like* in the hypothalamus of MO and mm control injected embryos. Total numbers of cells expressing H-GnRH and *oxytocin-like* in the hypothalamus in MO and mismatch control (mm) injected embryos. All embryos were fixed at 54±1hpf. *p*-values were obtained comparing MO versus mm controls using the Wilcoxon Rank Sum test. The count of total number of cells was obtained from both sides of embryo. All experiments were carried out at least 3 times for each MO. Min.: minimum number of cells per animal, max.: maximum number of cells per animal, n: number of animals analyzed, mm: control mismatch MO, MO: morpholino oligo.

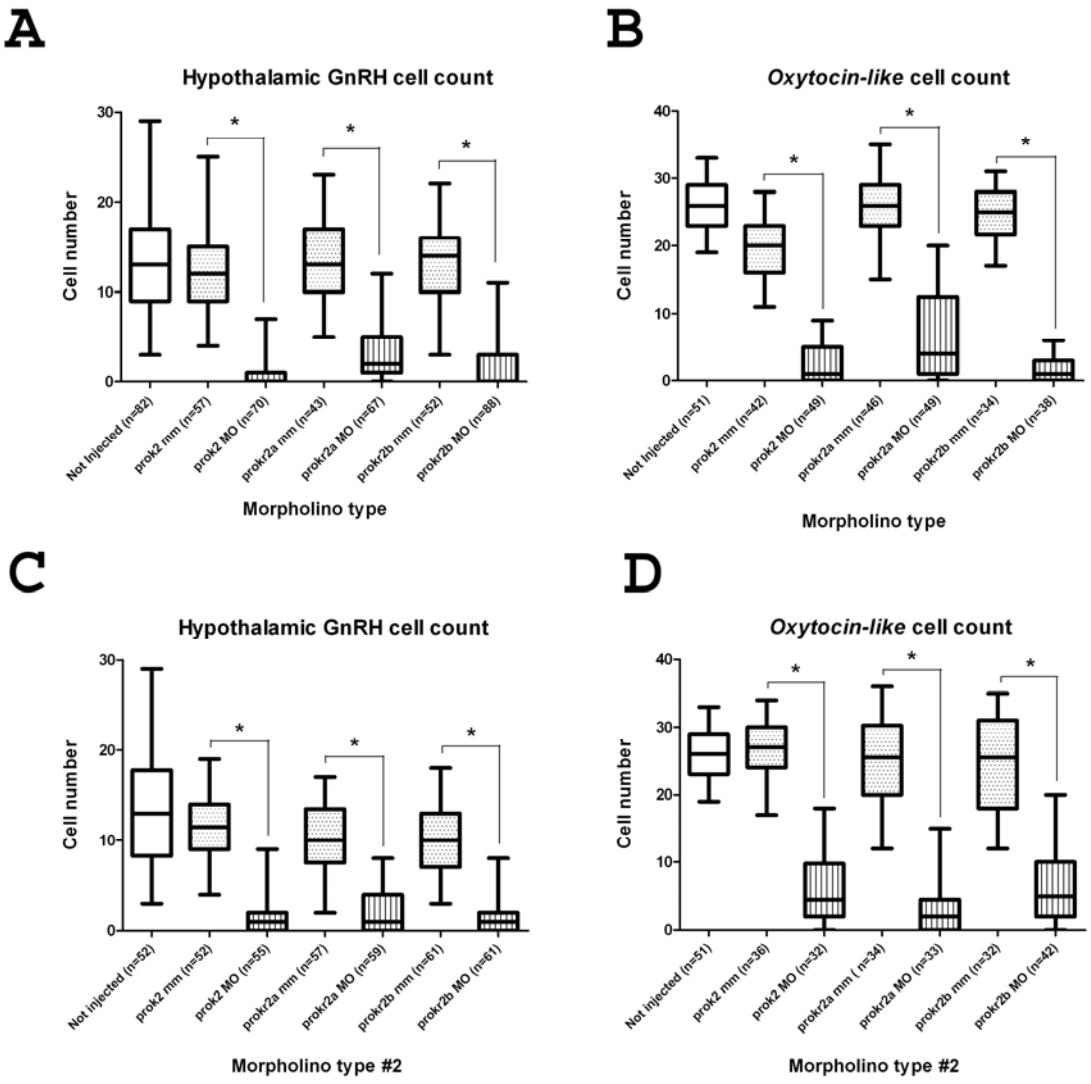


Figure 18. Two distinct hypothalamic cell types are reduced in number in MO injected embryos. Disruption of the *prokineticin2* signaling pathway causes a reduction in zebrafish hypothalamic cell types. Box and whisker plots summarizing counts of H-GnRH cells (**A**) and *oxytocin-like* cells (**B**). Loss-of-function of *prok2*, *prokr2a* and *prokr2b* genes with a second start site MO (Morpholino type #2) results in a similar phenotype to that observed with the first MOs in H-GnRH (**C**) and *oxytocin-like* cells (**D**). We tested the differences in cell counts between mismatch control embryos and MOs injected embryos using Wilcoxon Rank Sum test. We found a significant decrease in cell counts in *prok2*, *prokr2a* and *prokr2b* knockdown embryos ($p<0.0001$). n represents the number of animals analyzed (in parentheses). White, gray stiple, and gray line bars represent uninjected control, mm control, and MO treated embryos, respectively. mm: control mismatch MO. MO: morpholino injected animals.

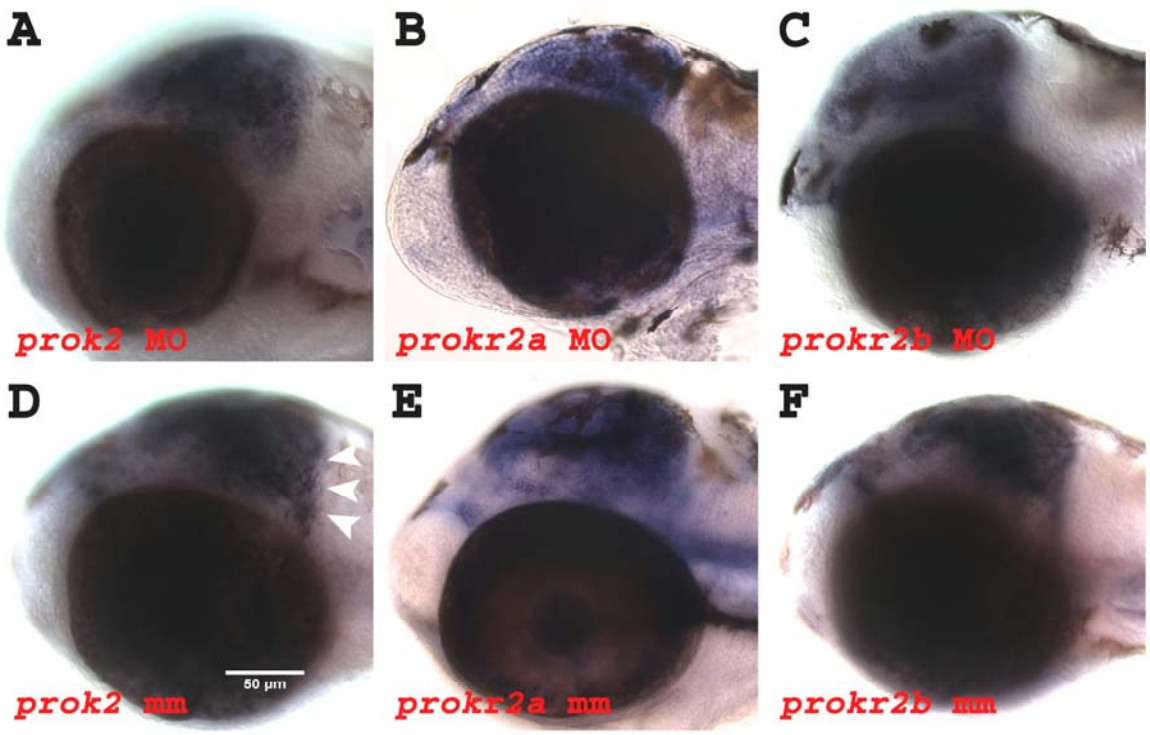


Figure 19. *otx2* gene is expressed in a normal spatio-temporal pattern in *prok2*, *prokr2a* and *prokr2b* morphants. Lateral views from 48±1hpf MO (**A-C**) or mismatch-injected (**D-F**) zebrafish embryos showing the expression of *otx2* in the midbrain hindbrain boundary (white arrowheads in **D**). We found no detectable alterations in the pattern of expression for *otx2* in the MO-injected embryos compared to the mm control animals. The posterior limit of expression (MHB) was not disrupted for this gene in both groups. Anterior to the left. mm: control mismatch morpholino, MO: morpholino oligo.

Finally, to assess early development of the midline signaling pathway in the *prok2*, *prokr2a* and *prokr2b* morphants, we used *sonic hedgehog* (*shh*) and *orthopedia* (*otp*) expression as landmarks to visualize the development of the midline tissue. *shh* plays an important role in the induction and early patterning of the hypothalamus (**Mathieu et al., 2002**) and *otp* is a key determinant of hypothalamic neural differentiation (**Wang & Lufkin, 2000**). We found that in the

MO treated embryos at 26±1hpf (**Figure 20A-C**) there is a disruption on the *shh* expression in the ventral diencephalon (white arrowheads in **Figure 20C**) compared to the control mm injected embryos (**Figure 20D-F**). In control animals the anterior domain of *shh* expression (corresponding to the developing hypothalamus) is observed as a solid and inverted U shape (black arrows in **Figure 20F**). In contrast, in the MO-injected embryos *shh* showed a decrease in expression along the ventral midline and a less defined posterior border of expression (white arrows in **Figure 20C**). *otp*, an hypothalamic marker, is normally expressed in the ventral hypothalamus and in the dorsal preoptic area (POA). *prok2*, *prokr2a* and *prokr2b* morphants (**Figure 20G-I**) showed a disrupted pattern of *otp* expression in the ventral hypothalamic expression domain (black arrowheads in **Figure 20I**) compared to the mm controls (**Figure 20J-L**) at 56±1hpf. Fewer *otp* expressing cells in the ventral hypothalamus are observed in the MO treated embryos compared to control mm MOs (white arrows in **Figure 20L**).

The data presented in this above suggest that *prokineticin2* signaling pathway genes play a role in the proper development and differentiation of the hypothalamus, and that a failure in the correct expression of this group of genes causes a loss of hypothalamic specific cell types.

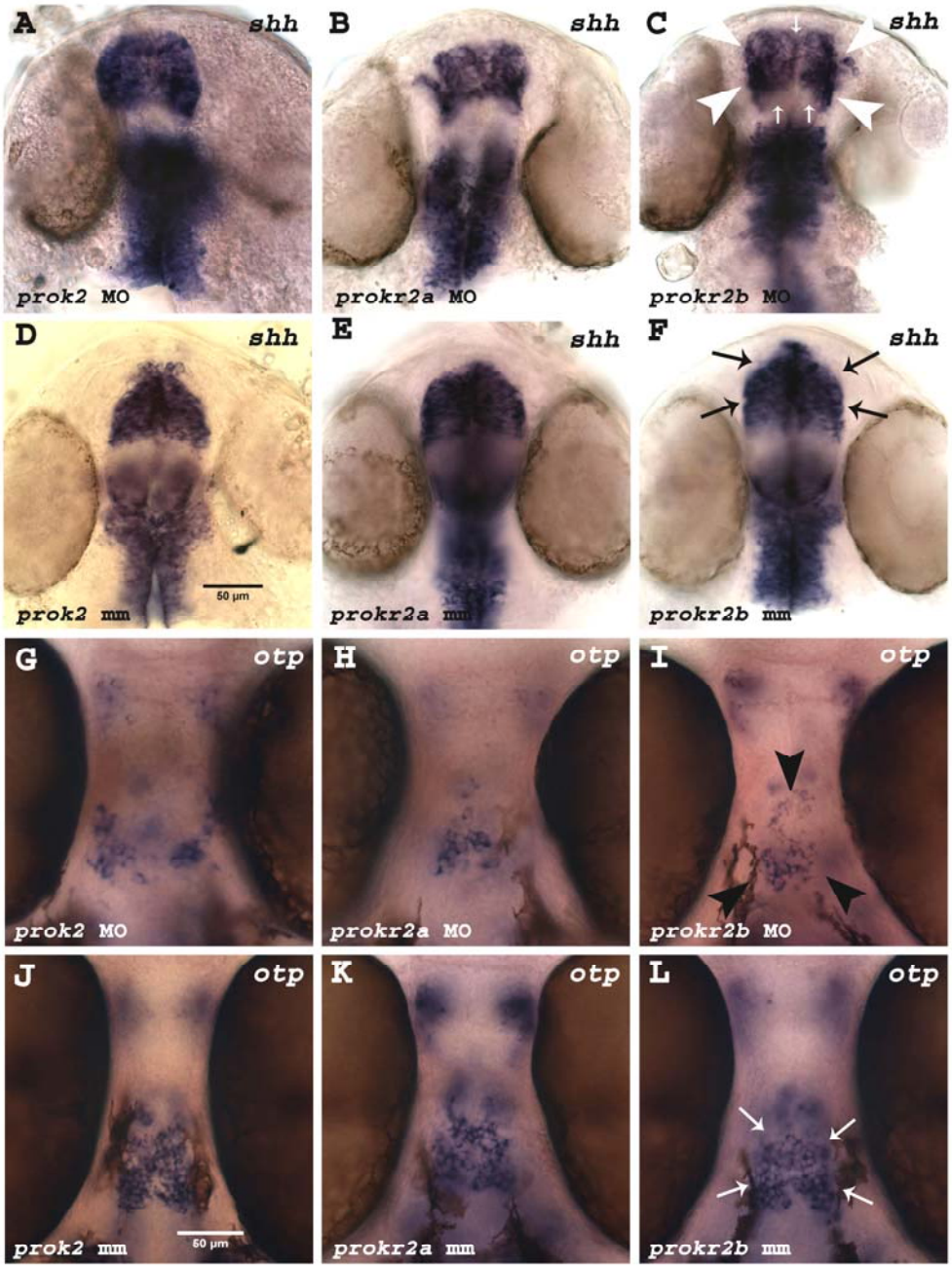


Figure 20. Loss-of-function of *prokineticin2* signaling pathway causes a disruption in the expression of hypothalamic markers. Expression of *shh* and *otp* genes is disrupted in *prok2, prokr2a* and *prokr2b* morphants. *sonic hedgehog* (**A-F**) at 26±1hpf and *orthopedia* (**G-L**) expression at 56±1hpf is disrupted in *prok2, prokr2a* and *prokr2b* MO injected embryos (**A-C**, white arrowheads for *shh* in **C**) (**G-I,** white and black arrowheads for *otp* in **H** and **I**, respectively) compared to the mm MO controls (**D-F**, black arrows in F) (**J-L**, white arrows in L). All pictures were taken from a ventral view with a 20X (**A-F**) or 40X (**G-L**) objective, anterior to the top. mm: control mismatch MO, MO: morpholino oligo.

3.3.4 Knockdown of *prok2*, *prokr2a* and *prokr2b* genes disturb anterior pituitary development

To determine whether the disruption in the development of the hypothalamus also affects adjacent anterior pituitary precursors, we used the POMC:GFP transgenic line to determine the effect of the knockdown of *prok2*, *prokr2a* and *prokr2b* genes on the development of the adenohypophysis. In this transgenic line, GFP-positive cells form a single cluster of cells in the anterior midline at 24hpf. Between 48 and 64hpf this cluster segregates into an anterior (rostral pars distalis) and a posterior (pars intermedia) group of cells (**Liu et al., 2003**). Knockdown of *prok2*, *prokr2a* and *prokr2b* genes caused a disruption in the normal pattern of GFP-expressing cells distribution (**Figure 21B-E**) between the anterior and posterior clusters compared to the control mm MOs (**Figure 21G-J**). The segregation between these two domains of cells observed in control embryos is lost in the MO treated animals (**Figure 21B-E,** white arrows). We found that the cells expressing POMC between these two clusters increase significantly in number (p<0.0001) in *prok2*, *prokr2a*, *prokr2b* morphants and when both receptor morpholinos are conijected compared to the mm control animals (**Figure 21K**).

Together, the data presented here demonstrate that the disruption of *prokineticin2* signaling pathway genes in zebrafish result in defects of hypothalamic, pituitary and olfactory tissues, suggesting that these genes are important for the correct patterning and/or differentiation of the developing telencephalon and diencephalon.

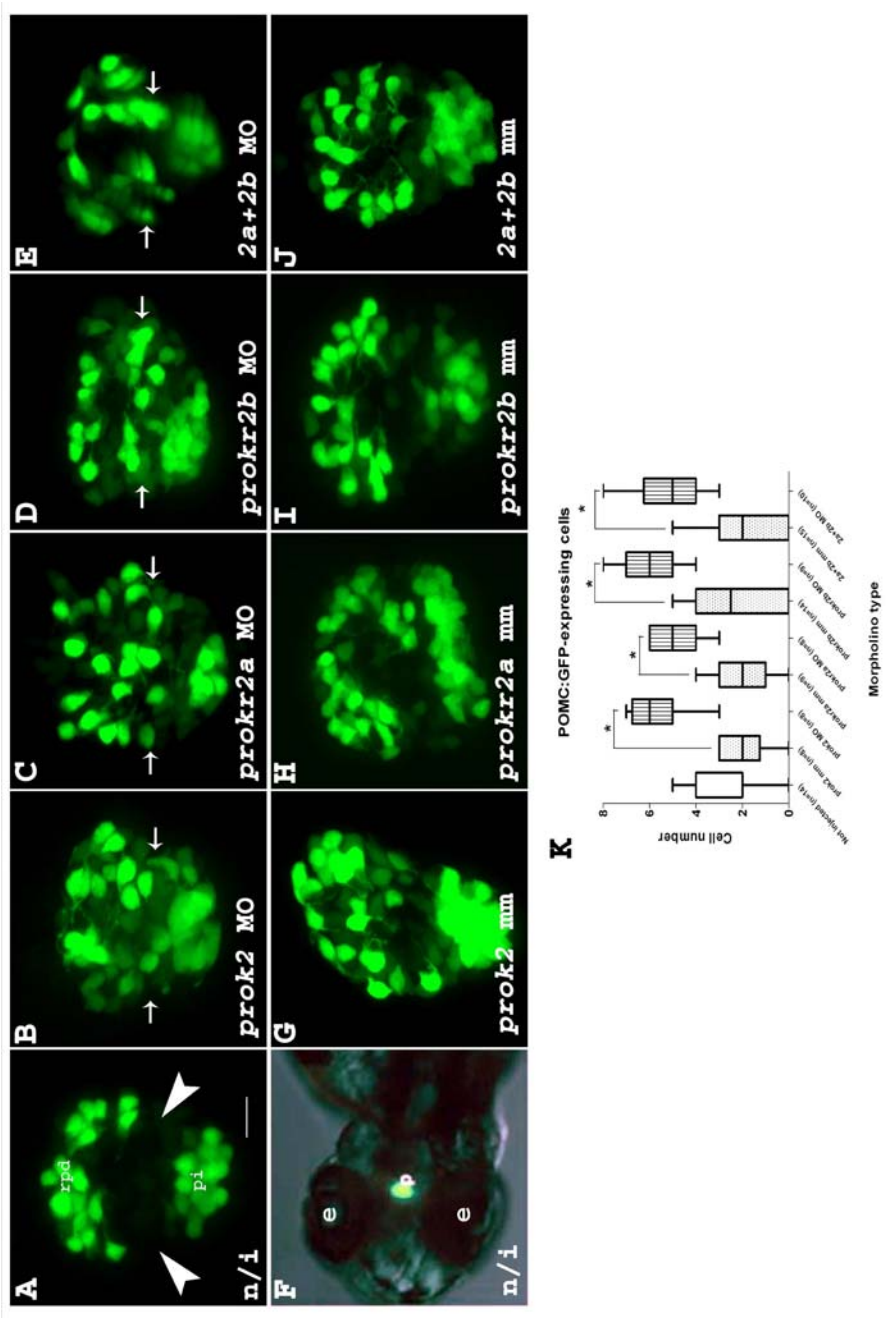


Figure 21. Knockdown of *prok2*, *prokr2a* and *prokr2b* proteins cause an abnormal pattern of GFP expressed in the pituitary at 72±1hpf in the POMC:GFP transgenic line. (**A**) Uninjected control (**B-E**) MO injected embryos, (**F**) representative ventral view of the anterior pituitary (p) (modified from **Liu et. al, 2003**) anterior to the left, (**G-J**) MO mismatch control animals. All pictures were taken from a ventral view with a 40X objective using an spinning disc microscope, anterior to the top. (**K**) Box and whisker plot summarizing count of POMC:GFP cells between the rostral pars distalis (top) and pars intermedia (bottom) adenohypophysis clusters of GPF-positive cells (white arrows in **B-E**). We found a significant increase in cell counts between these two clusters in *prok2*, *prokr2a*,

prokr2b and *prokr2a+prokr2b* knockdown embryos compared to the mm control (**p*<0.0001, Wilcoxon matched-pairs signed rank test). Number of analyzed animals is in parenthesis in **K**. White, gray stiple, and gray line bars represent uninjected control, mm control, and MO treated embryos, respectively. All experiments were performed at least two times for each treatment. POMC: proopiomelanocortin. Scale bar in A= 10μm. n/i: not injected, e: eye, p: anterior pituitary, rpd: rostral pars distalis, pi: pars intermedia.

4. DISCUSSION.

We have shown that the *prok2*-signaling pathway genes are conserved between mouse and zebrafish, and that, as has been reported in humans, decrement in function of these genes affects the development of the olfactory and hypothalamic tissues, both affected in KS patients.

4.1 *prokineticin2* signaling pathway gene expression.

Our results show the presence of two genes homologous to mouse *prokr2* and one for the *prok2* gene on the Zv9 version of the genome of *Danio rerio*. We found a high degree of synteny for *prok2* genes from mouse and zebrafish, both located in chromosome 6. Also the characteristics observed in zebrafish *prok2* (N-terminal AVITGA domain and ten cysteines residues), strongly suggest that this gene is highly conserved in vertebrate evolution. The divergence found between zebrafish PROKR2A and PROKR2B proteins in the N-terminal domain suggest that one of these GPCR could correspond to an homologous to mouse PROKR1, as this difference is conserved in mammal PROKRs (**Lin et al., 2002**), but thus far no PROKR1 has been documented in zebrafish. It is possible that PROKR2 gene may have duplicated over the course of evolution consistent with the whole genome duplication in the teleost fishes (**Amores et al., 1998**). According with this idea, the C-terminal portion of the second intracellular loop (IL2) of PROKR2 from human, which is essential for the interaction with cytoplasmatic G-proteins (**Peng et al., 2011**) and differs with PROKR1, is very similar for both zebrafish receptors (not

shown). Another possibility is that *prok2* reacts with both receptors to trigger different intracellular signaling pathways as seen in other species. *prok2* has higher affinity for both receptors in mammals (**Lin et al., 2002; Masuda et. al., 2002; Soga et al., 2002)**. This non-selectivity implies that the availability of PROK1/2 and PROKR1/2 determine which signaling cascade is triggered in a physiological or tissue specific fashion (**Negri et. al., 2007**). In addition, PROKR1 and PROKR2 can form heterodimers (**Marsango et al., 2010**) which could explain the similar phenotypes observed in the *prokr2a* and *prokr2b* gene knockdown experiments, suggesting that each receptor is necessary for *prok2* gene function and that *prokr2a* and *prokr2b* genes are not functionally redundant. In contrast, we observed that coinjection of *prokr2a* and *prokr2b* MOs in zebrafish embryos act synergistically to affect the formation of OSNs bundles in the OB. This phenotype suggest that both receptors are needed to establish the correct pattern of bundles in the OB, which suggests that *prokr2a* and *prokr2b* act together in a tissue specific manner to form medial, central and lateral bundles in the OB; but further analysis are needed to confirm this hypothesis. For additional exploration of the role of both receptors in zebrafish development, disruption of the *in silico* predicted zebrafish *prok1* gene is necessary.

In contrast to previously published reports in mouse, we could not detect expression of *prok2* and *prokr2* by *in situ* hybridization in the developing embryo, although we detected expression of these genes by RT-PCR. This method is comparatively much more sensitive than ISH suggesting that the mRNAs for these genes are found in low abundance in the zebrafish embryos. If the *prok2* gene is highly expressed in a few cells, then we should be able to detect them by ISH as

we have previously done for GnRH2 and TN-GnRH (or GnRH3) (**Gopinath et al., 2004; Whitlock et al., 2005a**) which are low abundance mRNAs, but highly expressed in a small number of cells. In contrast to the ligand, receptors are notoriously difficult to detect using DIG labeled probes (as opposed to radio-labeled probes) thus we were not surprised that we were unable to detect *prokr2a* and *prokr2b* by ISH in the adult brain and embryos. As a result, at this time we do not know the precise expression domains of the *prok2*, *prokr2a* and *prokr2b* genes in the embryo, but our RT-PCR microdissection analysis show that these genes are expressed in the olfactory and ventral diencephalic regions of the embryo. In agreement with these findings, the results of our gene knock-down experiments show that these genes play a role in the formation of the developing OB and hypothalamus.

Using ISH and PCR we were able to detect *prok2* in adult hypothalamus a region previously described to contain *prok2*-expressing cells in mammals (**Cheng et al., 2006**). In contrast to mouse (**Ng et al., 2005, Cheng et al., 2006, Puverel et al., 2009**) we found no evidence for *prok2* expression in sections of the adult OB, but by RT-PCR and qPCR analysis, we did detect expression in the telencephalic region but with a lower relative abundance than in the ventral diencephalon. ESTs profiles (Unigene database) show that zebrafish *prok2*, *prokr2a* and *prokr2b* genes are expressed only in reproductive adult tissue (not shown) as seen in rodents and humans (for review see **Martin et al., 2011**). Concurrently with our study, Ayari and colleagues have cloned zebrafish *prok2* and shown by *in situ* hybridization that it is expressed throughout the adult brain: ventral and dorsal telencephalon, midbrain–forebrain boundary, rostral and rostro-ventral midbrain, diencephalon,

mesencephalon, optic tectum, cerebellum, dorso-caudal hindbrain, and in the ventral part of the pons (**Ayari et al., 2010**). The discrepancies of *prokineticin2* expression pattern in the zebrafish adult brain described need to be resolved, but as the experimental procedures used were similar in both cases (**Soussi-Yanicostas N, *personal communication***), further analysis, like the generation of a specific antibody for zebrafish PROK2, must be accomplished. Nevertheless taking into account that we replicated our expression pattern of *prok2* with the probe used by Ayari and collagues and that our real time and semiquantitative PCR analysis are in accordance with our *prok2* ISH pattern of expression, we presume that the expression of *prok2* observed in Ayari paper is because of an experimental error/counter staining of the samples. Considering that in this study the majority of neurons in the brain express *prok2*, a pattern dissimilar to the precise and restricted domains of expression observed in mammals, we are confident in the validity of our results.

4.2 Knockdown effects of *prokineticin2* signaling pathway.

4.2.1 Olfactory system.

Knockdown of *prok2*, *prokr2a* and *prokr2b* genes causes a reduction in olfactory bulb size, affects axon terminations in the OB and totally abolishes the expression of *emx1*. The abnormal shape of the OSN axonal projections to the developing OB in the morphant embryos may affect synaptic connections in the olfactory system between OSNs axons and dendrites of OB neurons as has been suggested for the

knockdown of other KS related genes in zebrafish (**Yanicostas et al., 2009**). Although in our knockdown experiments the OSNs exit the OE, grow toward CNS in fascicles and desfasciculate tangentially on the surface of the OB to form the three main axon bundles (medial, center and lateral) (**Miyasaka et al., 2005; Vitebsky et al., 2005**), our results suggest that the axonal connections in target glomeruli are perturbed. Further analysis that reveal the integrity of the proto-glomerular condensations organization and postsinaptic densities are needed. Coinjection of both receptor MOs disrupted the stereotyped pattern of axonal endings in the developing OB which suggests that both receptors are needed for the correct formation of the three bundles of axonal projections in the OB. It is possible that the abnormalities seen in our knockdown experiments are due to a failure in the proper segregation of axon termination within the OB, but problems with the OSNs itself can not be discarded. Analysis of the number of OSNs and the integrity of pioneer OSNs axons (**Whitlock & Westerfield, 1998**; **Vitebsky et al., 2005**) must be assessed to confirm this hypothesis. The phenotype we observed in the OMP:YFP transgenic line may result from the fact that the target tissue of the OSNs axons is disrupted and making this failure secondary to OB disgenesis. Furthermore *emx1*, a transcription factor involved in the development of the anterior telencephalon in vertebrates (**Morita et al., 1995, Kawahara & Dawid 2002**), required for the formation of olfactory circuitry in *Drosophila melanogaster* (its expression is necessary for precise targeting of projection neurons dendrites to appropriate glomeruli) (**Lichtneckert et al., 2008**), and suggested to guide pioneer axons from the OP to the OB in zebrafish (**Whitlock and Westerfield, 1998**), is not expressed in the OB of *prok2*, *prokr2a* and *prokr2b* morphants.

emx1 is a downstream target of the *gli3* gene (**Theil et al., 1999, Tole et al., 2000**), which is part of the *sonic hedgehog-patched-gli* signalling pathway. In mouse, *shh* is expressed in the early appearing protoglomeruli during development, and is involved in the entry, growth, and branching of OSN axons into OB glomeruli (**Gong, et al., 2009**). As the expression of *shh* in the developing forebrain (which originates from the anterior neural plate) is sligthly affected in our knockdown studies, the *emx1* lack of expression observed in *prok2*, *prokr2a* and *prokr2b* morphants could be a result of this mild failure or the effect on *emx1* may be independent of *shh* signaling. Thus far, no information about a relation between *emx* and *prok2/prokr2* genes have been described.

The *vax1* gene, which is a homeobox gene closely related to *emx*, is required downstream of *shh* in the differentiation of the basal forebrain in mice and *Xenopus* (**Hallonet et al., 1999**). In addition, mutations of *vax1* cause holoprosencephaly and craniofacial defects (**Hallonet et al., 1999**) as seen in *shh* mutants (**Belloni et al. 1996; Chiang et al. 1996**). Therefore, both mutated genes result in abnormal development of the basal forebrain. Although in mouse and *Xenopus*, *vax1* is detected after anterior neural plate specification/determination, it could function later in the specification or maintenance of ventral anterior domains in the forebrain (**Hallonet et al., 1999**). These results from mouse and *Xenopus* suggest that *emx1* may act as *vax1* to control anterior telencephalon development.

4.2.2 Hypothalamus.

In addition to the olfactory system phenotype, depletion of the zebrafish *prokineticin2* signaling pathway results in a significant reduction in the number of H-

GnRH and *OT-like*-expressing cells in the hypothalamus and a disruption on the expression of *shh* and *otp* genes on hypothalamic domains. *shh* signaling pathway plays an essential role in the patterning of the ventral CNS and disruption of this cascade have been suggested to downregulate (**Sarnat & Flores-Sarnat, 2001**) and/or modulate (**Del Giacco et al., 2006**) *otp* expression in the hypothalamus. Also, *shh* promote hypothalamic fates and cooperates with *nodal* to mantain hypothalamic domains (**Mathieu, et al., 2002**), making this early signal essential for the induction and establishment of the hypothalamus in zebrafish. In our knockdown studies, hypothalamic H-GnRH and *oxytocin-like* cells are missing. This result suggests that the *prok2* signaling pathway may be involved in the proper development of the hypothalamus, possibly acting early in the induction/establishment of the ventral diencephalon in concert with the *shh* pathway as seen in the development of the enteric nervous system (see below). Another possibility is that *prok2* signaling may function later in specification/maintenance of ventral diencephalon domains in the forebrain. Although so far there is no evidence of an interaction between *shh* and *prokineticin* to control the development of CNS structures, both genes are proposed to act together in the development of enteric nervous system. *shh* controls the proliferation and differentiation of neural crest cells (NCCs) and modulates the effect of glial cell-line derived neurotrophic factor (GDNF) over NCCs in mouse (**Fu et al, 2004**) and zebrafish (**Reichenbach et al., 2008**). Also, *prok1* modulates proliferation and differentiation of enteric NCCs (**Ngan et al., 2007**). In addition, *prokineticins* work coordinately and can compensate the loss of GDNF/Ret signaling (which are essential for enteric NCCs development) in Hirschsprung disease modulating differentiation and proliferation of

NCCs (**Ngan et al., 2008**). In mouse, *prokineticins* and their cognate receptors have been detected as early as stage E7 in mouse embryos (**Ngan et al., 2007**), which suggest that *prokineticin* signaling is involved in early developmental processes, though their roles during embryonic development are yet to be elucidated. This is not a surprise as new functions of the *prokineticin* pathway are emerging fast (see **Ngan & Tam, 2008** for review). Furthermore the role of *prokineticin2* in GnRH cell and olfactory system development is not well understood as mutations are also seen in normosmic GnRH deficiency. This suggests a role of *prokineticin2* beyond neuronal migration as GnRH neurons do not express PROKR2 (**Martin et al., 2011**).

Analysis of other hypothalamic cell subtypes (dopaminergic and vasopressin expressing cells for example) in our knockdown experiments is needed to confirm the role of the *prokineticin2* pathway in the hypothalamic neurosecretory system development.

4.2.3 Pituitary.

We showed that the loss of function of *prok2*, *prokr2a* and *prokr2b* genes affect the correct formation of the pituitary gland, which show a scattered rather than a strict and stereotyped domain of expression in the POMC:GFP transgenic line at 3dpf. This results are in accordance with recent reports of partial defects at the level of the adenohypophysis for KS and IHH patients (**Sykiotis et al., 2010; Correa et al., 2011**). Coinjection of both receptor MOs causes no additional phenotypes compared with the single morphants, which suggest that in zebrafish both

prokineticin2 receptors are redundant in their role in adenohypophysis development.

BMPs, FGFs and SHH are signal molecules required to organ commitment, early patterning, and positional determination in pituitary early development through the induction of downstream transcription factor expression. (**Burgess et al., 2002, Dasen & Rosenfeld, 1999**). Studies using the overexpression of hedgehog inhibitor HIP (hedgehog interacting protein), revealed that hedgehog signaling is necessary for proliferation and specification of the pituitary ventral domain (**Treier et al., 2001**). In zebrafish, *shh* from the ventral diencephalon plays a crucial role in the induction, patterning and growth of the zebrafish adenohypophysis and hypothalamus (**Herzog et al., 2003**). In addition, the *you-too* zebrafish mutant, which is defective in the *gli2* gene (downstream effector of hedgehog signaling), cause defects in pituitary and hypothalamus development (**Kalstrom et al., 1999**). In our knockdown studies, the effect observed in pituitary development was mild, a result consistent with the subtle disruption of *shh* in the anterior midline at 26±1hpf observed in the morphants. Despite this result, we can not discard a direct effect of *prok2* signaling over pituitary development. Further analyses are required to dissect the effect of *prok2* signaling over anterior pituitary formation.

4.2.4 Kallmann syndrome.

Knockdown of *kal1a* in medaka (*Orizyas latipes*) resulted in a disruption of GnRH neuronal migration (**Okubo et al., 2006**), and in zebrafish cause the loss of endocrine GnRH cells of the hypothalamus, but not of neuromodulatory GnRH cells of the midbrain and terminal nerve (**Whitlock et al., 2005a**). The similarities of the

prok2/*prokr2*, *kal1a* and *fgfr1* (**Kim et al., unpublished results**) knockdown phenotypes suggest these genes may act in a common signaling pathway controlling H-GnRH cell differentiation. Indeed, both *fgfr1* and *anosmin1* (an ortholog gene of zebrafish *kal1a*) need heparan sulfate proteo-glycans (HSPGs), to exert their normal biological function (**Guimond & Turnbull, 1999; Soussi-Yanicostas et al., 1996**) and these three components physically interact to form a protein complex (**González-Martínez et al., 2004**). HSPGs are essential components of the extracellular matrix and the cell surface membrane (**Zhang et al., 2009**) and interact with a variety of cellular proteins, including growth and differentiation factors, morphogens, extracellular matrix components and regulating ligand receptor interactions (see **Bernfield et al. 1999** for review). For instance, HSPGs modulate important regulatory proteins such as WNT, BMP, FGF, and SHH, and modify the signal and/or regulate receptor activation (**Fico et al., 2011**). HSPGs can affect the receptor activation by capturating secreted factors after their withdrawal increasing their concentration/availability, regulating morphogen movement, signaling and trafficking or might stabilize ligand–receptor interactions (**Yan & Lin, 2009**; **Fico et al., 2011**). In the fruit fly *D. melanogaster*, HSPGs modulates Hedgehog signaling during embryogenesis, and are required for its movement in the developing wing imaginal disc (**Han et al., 2004**). Prokineticins also binds to HSPGs (**LeCouter et al., 2003**) as other Kallmann related genes. Also, the *heparan sulfate 6-O-sulfotransferase 1* enzyme (which modifies HSPGs) is mutated in patients with IHH (**Tornberg et al., 2011**). Finally, HSPGs are found in the early developing nervous system in mouse (**Luxardi et al., 2007**) and manipulations in vertebrate embryos have provided insight into their role in brain

patterning (**Galli et al., 2003**). So, it is possible that *kal1a*, *fgfr1* and *prok2*/*prokr2* genes acts in concert to control the development of GnRH cells in the developing hypothalamus.

Using yeast two-hybrid screens, Kim and collagues (**Kim et al., 2010**) found that the *emx1* gene (that is strongly affected in our knockdown experiments) interacts with the WDR11 protein, which is a novel mutation found in KS patients (**Kim et al., 2010**). *wdr11* is expressed in the entire developing central nervous system of the mouse embryo and in the olfactory bulb, piriform cortex, cerebellum, hypothalamus and hippocampal region of the mouse adult brain (**Kim et al., 2010**; **Kim & Layman, 2011**). In zebrafish the expression of *wdr11* is broadly expressed in the forebrain, midbrain and hindbrain at 24hpf partially overlapping with *emx1* domains of expression (**Kim et al., 2010**; **Kim & Layman, 2011**). The role of WDR11 in KS suggests a possible connection between this disease and the *shh* pathway, as *emx1* (which colocalizes with WDR11 *in vivo* and *in vitro*) is part of the *shh-patched-gli-emx* signaling pathway (**Kim et al., 2010**).

4.2.5 Current model of forebrain specification.

In the development of the anterior nervous system, morphogen signals are translated into transcription factor codes that allow induction of different forebrain domains, which finally leads to the differentiation of distinct neurons in diverse territories of the telencephalon (**Takahashi & Lui, 2006**). The mechanism regulating the interaction between *wnt*/*bmp*, *fgf* and *shh* morphogens in the correct development of the forebrain are still not elucidated in detail. However *wnt*, *bmp*, *fgf*, and s*hh* pathways show positive and negative feedback regulations in diverse

structures and at different developmental stages (**Bertrand & Dahmane, 2006**). *shh* is necessary and sufficient for induction of the ventral telencephalon, while loss of *shh* function expand dorsal telencephalic markers as *emx1* in mice (**Chiang et al., 1996; Ohkubo et al., 2002**). A correct balance between *fgf* and *bmp*/*wnt* pathways is required for induction of rostral and dorsal telencephalic domains, respectively (**Kuschel et al., 2003**). In addition, *fgf* and *shh* pathways may act together in the control of ventral telencephalon patterning as both factors act coordinately to form the anterior forebrain in vertebrates (**Shanmugalingam et al., 2000; Crossley et al., 2001; Ohkubo et al., 2002**). Furthermore, *shh* and *fgf* are repressed by the *bmp* pathway in the dorsal forebrain (**Ohkubo et al., 2002**).

Mouse mutant in the zinc finger transcription factor *gli3* shows a disrupted formation of the pallium, olfactory bulbs, hippocampus and the dorsal boundary between the diencephalon and telencephalon (**Franz, 1994; Theil et al., 1999**) between other forebrain structures. In this mutant there is a loss of *emx1* and *emx2* genes in the dorsal forebrain and an expansion and reduction of *fgf8* and *bmp4* expression pattern, respectively (**Theil et al., 1999; Kuschel et al., 2003**). These studies place *gli3* upstream of *emx* genes in the genetic pathway controlling dorsal telencephalic patterning. In addition, *emx2* expression is regulated in the dorsal telencephalon directly by *wnt* and *bmp,* and loss of the two latter genes coincides with a severe reduction of *emx2* expression in the dorsomedial telencephalon (**Theil et al., 1999; Tole et al., 2000; Theil et al., 2002**). This finding places *emx2* downstream of *wnt* and *bmp* in the control of telencephalic specification. The results discussed above suggest that *bmp*, *wnt*, *gli3* and *emx2* genes may act together in the same genetic pathway to control dorsal telencephalic development

(**Theil et al., 2002**). Morphogen target genes like *pax6* and *ngn2* are reduced in the dorsal telencephalon, and *mash1* and *dlx2* ventral markers shows a ectopic expression in the dorsal telencephalon, suggesting a ventralization of this structure in the *gli3* mutant (**Kuschel et al., 2003**). Thus, the *gli3* gene is essential for the establishment and maintenance of the dorsal signaling center (**Kuschel et al., 2003**). Furthermore, *gli3* and *wnt* genes are not affected in the *emx2* mutant, showing that these genes are not under the control of *emx2* (**Yoshida et al., 1997**).

fgf8 is expressed in the rostromedial pole of the telencephalon, where it controls the development of the forebrain in zebrafish (**Shanmugalingam et al., 2000**) and mice (**Meyers et al., 1998**). Loss of function of *fgf8* causes a reduction of the forebrain and downregulate genes that are expressed in the subpallial region. In zebrafish, loss of *fgf* signaling pathway lead to defects in telencephalic and diencephalic ventral patterning. *shh* is dependent in *fgf* signalling in the hypothalamus and the zona limitans intrathalamica (**Walshe & Mason, 2003**) and the expression of *fgf* signaling is under the control of *hh* morphogen in the forebrain (**Miyake et al., 2005**). These interactions are essential for proper forebrain patterning and places *fgf* signaling downstream of *shh* for the induction of this important region of the brain. Moreover, in explant cultures assay, *fgf8* induced *mash1* and *dlx2* expression and suppressed *emx1* transcription (**Kuschel et al., 2003**). In addition, there is an antagonism between *fgf8* from the anterior telencephalon and *wnt/bmp* from the dorsal telencephalon, suggesting that *fgf8* plays an important role in the ventralization of the telencephalon phenotype observed in the *gli3* mutant (**Kuschel et al., 2003**). In gain and lost of function experiments using mouse embryos, Okada and colleagues (**Okada et al., 2008**)

found that overexpression of *fgf8* up-regulated transcription factors *vax1* and *zic2* in midline structures and inhibition of *fgf* signalling decrease the expression domain of those genes. In *shh* null embryos (where *fgf8* is expression is reduced), *vax1* and *zic2* are downregulated and ectopic expression of *fgf8* in this mutant, recovered those midline transcription factors (**Okada et al., 2008**). In addition, *gli3* mutant mice showed high levels of *vax1* and *zic2* and blockage of *fgf* pathway decreased their expression at the midline. Interestingly, ectopic *shh* expression in *gli3* mutant only upregulated some of the midline transcription factors, suggesting that *fgf8* signaling may participate in midline structures development independently of *shh* action (**Okada et al., 2008**).

Recently, the *sp8* gene was found to be expressed along the medial lateral axis of the forebrain, showing a gradient with higher expression at caudomedial levels compared to rostrolateral regions. According with this, KO embryos for *sp8* displayed olfactory bulbs and septum dysgenesis and complete absence of the midline (**Zembrzychi et al., 2007**), suggesting that *sp8* is required for early patterning of the forebrain. In the *sp8* deficient mice, expression of dorsally expressed *pax6* is disrupted and *emx2* transcription is expanded compared to wild-type embryos, causing a caudalization of the cortical areas (**Zembrzychi et al., 2007**). In addition, *fgf8* expression is affected in *sp8* mutant embryos, consistent with the depressing role of *emx2* in *fgf8* expression (**Fukuchi-Shimogori & Grove 2003**). *shh* and *wnt* expression remains unchanged in the absence of *sp8*, which suggest the *sp8* may be required for the *fgf8* activity and that the forebrain developmental failure observed in mutant embryos is independent of *wnt* and *shh* signaling pathways (**Zembrzychi et al., 2007**). Furthermore, *emx2* or *pax6* function

does not affect *sp8* expression, suggesting that *sp8* acts upstream of these genes (**Zembrzychi et al., 2007**). *emx2* and *pax6* transcription factors are expressed in opposed gradients trough the anterior posterior axis of the forebrain (high at caudomedial and low at rostrolateral positions for *emx2*, and the opposite gradient for *pax6*), with mice deficient in *emx2* and *pax6* showing disruption of dorsal telencephalon structures where those genes are normally expressed (**O´Leary & Nakagawa, 2002**). Furthermore, reduced levels of ventrally expressed transcription factors like *mash1*, *gsh2*, *nkx2.1*, and *dlx1* is observed in *sp8* KO embryos (**Zembrzychi et al., 2007**).

Mice defective in retinoic acid production (lacking a retinal dehydrogenase enzyme) present strong defects in forebrain development by controlling *fgf* and *shh* signaling and in turn reduce dorsal *emx2* and ventral *nkx2.1* telencephalic markers (**Ribes et al., 2006**). In chick embryos with reduced retinoic acid signaling, *shh* and *fgf8* genes are highly reduced, suggesting that these two pathways mediate retinoic acid action in forebrain development (**Schneider et al., 2001**).

Here we showed that the knockdown of *prok2* signaling produces a mild effect over *shh* expression in the anterior ventral midline and that *emx1* expression is totally abolished in the morphant embryos. This result suggest that the slight disruption of *shh* expression caused by *prok2* signaling knockdown (by an unknown mechanism) affect directly *emx1* or more likely *prok2* signaling knockdown affect *emx1* (and probably also *otp*) by a mechanism independent of *shh*. This would implicate that *emx1* and *otp* genes may act in the specification or mantainance of forebrain structures after anterior neural plate induction. Thus, knockdown of *prok2* signaling may affect cells that express *shh* in the ventral diencephalon by a

mechanism not related to *shh* signaling (maybe the cells that do not express *shh* in the morphants are affected directly or indirectly by *prok2* signaling, compromising their fate as *shh* expressing cells in this particular region). Analisys of the expression of other patterning genes in the morphants (such as *fgf8* or *gli3*) is required to dissect the precise role of *prok2* signaling in the development of the olfactory system and ventral midline structures in zebrafish embryos.

All the data presented above and the abnormal expression of *shh*, *otp* and *emx1* observed in our *prok2*, *prokr2a*, and *prokr2b* knockdown experiments suggest that *prokineticin2* signaling pathway genes may play a role in the correct patterning and/or differentiation of the ventral and dorsal forebrain cellular domains where the olfactory, hypothalamic and pituitary tissues come from (**Whitlock & Westerfield, 2000**) (**Figure 22**).

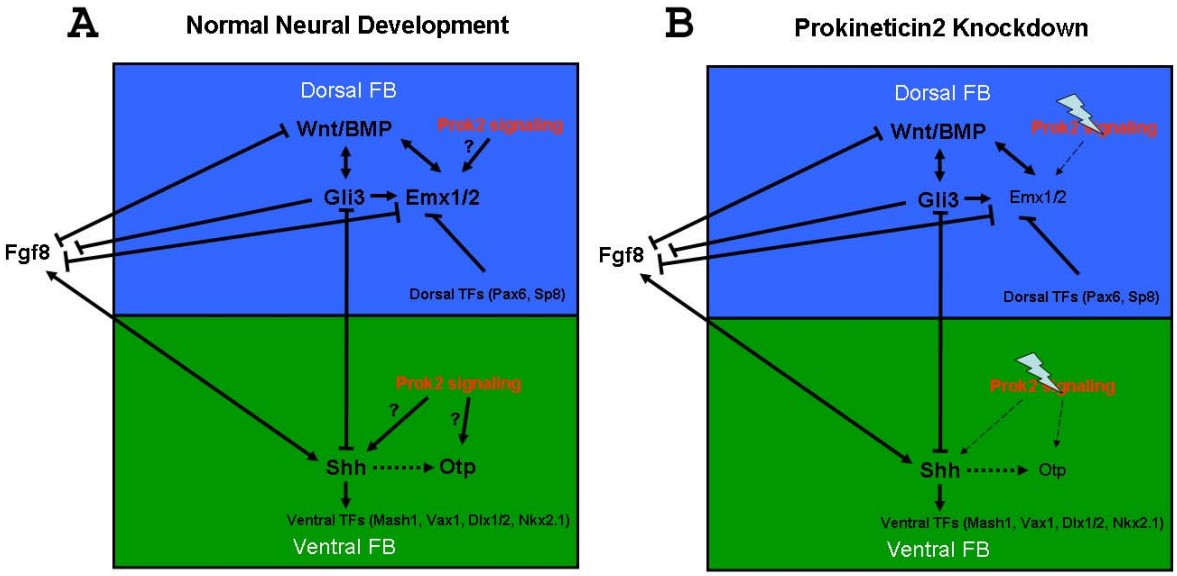


Figure 22. *prok2* signaling pathway affect generation of forebrain derivated tissues.
Proposed model for the role of *prok2* signaling pathway in controlling forebrain tissue development. In wild type embryos (**A**), *shh*, *emx1*, and *otp* are expressed normally to induct forebrain structures, but when *prokineticin2* signaling pathway is

disrupted (**B**), *shh*, *emx1*, and *otp* genes are downregulated by an unknown mechanism (?) causing midline forebrain failures (in hypothalamic and pituitary tissues) and rostral telencephalon development (olfactory system). The action of *prokineticin2* over genes essential for proper development of the nervous system (*fgf8*, *gli3*, *wnt*/*bmp*) remains to be elucidated. Arrows represent positive influences, T bars negative ones, and the dashed line a proposed interaction. FB: Forebrain, TFs: Transcription factors.

5. CONCLUSION.

Taken together, our data suggest that the *prok2/prokr2* zebrafish genes are important for the normal development of the olfactory system, hypothalamus, and the anterior pituitary. The specific role of this versatile pathway in the development of the zebrafish forebrain remains to be elucidated.

6. REFERENCES.

- Abreu AP, Barbosa Trarbach E, de Castro M, Frade Costa EM, Versiani B, Matias Baptista MT, Mendes Garmes H, Bilharinho Mendonca B, Latronico AC. (2008). Loss-of-Function Mutations in the Genes Encoding Prokineticin-2 or Prokineticin Receptor-2 Cause Autosomal Recessive Kallmann Syndrome. *Journal of Clinical Endocrinology and Metabolism*. 93(10):4113–4118.
- Amores A, Force A, Yan YL, Joly L, Amemiya C, Fritz A, Ho RK, Langeland J, Prince V, Wang YL, Westerfield M, Ekker M, Postlethwait JH. (1998). Zebrafish *hox* Clusters and Vertebrate Genome Evolution. *Science*. 282:1711-1714.
- Ardouin O, Legouis R, Fasano L, et. al. (2000). Characterization of the two zebrafish orthologs of the KAL1 gene underlying X chromosome linked KS. *Mechanisms of Development.* 90(1):89-94.
- Ayari B, El Hachimi K, Yanicostas C, Landoulsi A, Soussi-Yanicostas N. (2010). Prokineticin 2 expression is associated with neural repair of injured adult zebrafish telencephalon. *Journal of Neurotrauma*. 27:959-972.
- Bernfield M, Götte M, Park PW, Reizes O, Fitzgerald ML, Lincecum J, Zako M. (1999). Functions of cell surface heparan sulfate proteoglycans. *Annual Review of Biochemistry*. 68:729–777.
- Bertrand N, Dahmane N. (2006). Sonic hedgehog signaling in forebrain development and its interactions with pathways that modify its effects. *Trends in Cell Biology*. 16(11):597-605.
- Belloni E, Muenke M, Roessler E, Traverso G, Siegel-Bartelt J, Frumkin A, Mitchell

HF, Donis-Keller H, Helms C, Hing AV, Heng HHQ, Koop B, Martindale D, Rommens JM, Tsui LC, Scherer SW. (1996). Identification of *Sonic hedgehog* as a candidate gene responsible for holoprosencephaly. *Nature Genetics.* 14:353-356.

- Burgess R, Lunyak V and Rosenfeld MG. (2002). Signaling and transcriptional control of pituitary development. *Current Opinion in Genetics & Development*. 12:534–539.
- Cadman SM, Kim SH, Hu Y, González-Martínez D, Bouloux PM. (2007). Molecular Pathogenesis of Kallmann's Syndrome. *Hormone Research*. 67:231–242.
- Cariboni A, Maggi R (2006). Human Genome & Diseases: Review Kallmann's syndrome, a neuronal migration defect. *Cellular and Molecular Life Science.* 63:2512–2526.
- Chen J, Kuei C, Sutton S, Wilson S, Yu J, Kamme F, Mazur C, Lovenberg, Liu C. (2005). Identification and Pharmalogical characterization of prokineticin 2β as a selective ligand for prokineticin receptor 1. *American Society for Pharmacology and Experimental Therapeutics*. 67:2070-2076.
- Cheng MY, Leslie FM and Zhou QY. (2006). Expression of Prokineticins and Their Receptors in the Adult Mouse Brain. *Journal of Comparative Neurology*. 498(6):796-809.
- Chiang C, Litingtung Y, Lee E, Young KE, Corden JL, Westphal H, Beachy PA. (1996). Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function. *Nature.* 383:407-413.
- Cole LW, Sidis Y, Zhang C, Quinton R, Plummer L, Pignatelli D, Hughes VA, Dwyer AA, Raivio T, Hayes FJ, Seminara SB, Huot C, Alos N, Speiser P, Takeshita A, Van

Vliet G, Pearce S, Crowley WF Jr, Zhou QY, Pitteloud N (2008). Mutations in prokineticin 2 and prokineticin receptor 2 genes in human gonadotrophin-releasing hormone deficiency: molecular genetics and clinical spectrum. *Journal of Clinical Endocrinology and Metabolism*. 93(9):3551-9.

- Correa FA, Tusset C, Abreu AP, Latronico AC, Carvalho LR, Brito VN, Arnhold IJP, Mendoca BB. (2011). Identification of two variants in PROKR2 gene in a cohort of patients with congenital hypopituitarism. *Endocrine Reviews*. P1-279.
- Coumoul X, Deng CX. (2003). Roles of FGF receptors in mammalian development and congenital diseases. *Birth Defects Research*. 69(4):286-304.
- Cox KH, DeLeon DV, Angerer LM, Angerer RC. (1984). Detection of mRNAs in sea urchin embryos by in situ hybridization using asymmetric RNA probes. *Developmental Biology*. 101(2):485-502.
- Crossley PH, Martinez S, Ohkubo Y, Rubenstein JL. (2001). Coordinate expression of Fgf8, Otx2, Bmp4, and Shh in the rostral prosencephalon during development of the telencephalic and optic vesicles. *Neuroscience*. 108: 183–206.
- Dasen JS, Rosenfeld MG. (1999). Signaling mechanisms in pituitary morphogenesis and cell fate determination. *Current Opinion in Cell Biology*. 11:669–677.
- Del Giacco L, Sordino P, Pistocchi A, Andreakis N, Tarallo R, Di Benedetto B, Cotelli F. (2006). Differential regulation of the zebrafish *orthopedia1* gene during fate determination of diencephalic neurons. *BMC Developmental Biology*. 6:50-69.
- De Roux N. (2005). Isolated Gonadotropic Deficiency with and without Anosmia: A Developmental Defect or a Neuroendocrine Regulation Abnormality of the

Gonadotropic Axis. *Hormone Research*. 64:48-55.

- Dereeper A, Guignon V, Blanc G, Audic S, Buffet S, Chevenet F, Dufayard JF, Guindon S, Lefort V, Lescot M, Claverie JM, Gascuel O. (2008). Phylogeny.fr: robust phylogenetic analysis for the non-specialist. *Nucleic Acids Research*. 36:W465–W469.
- Dodé C, Levilliers J, Dupont JM, De Paepe A, Le Dû N, Soussi-Yanicostas N, Coimbra RS, Delmaghani S, Compain-Nouaille S, Baverel F, Pêcheux C, Le Tessier D, Cruaud C, Delpech M, Speleman F, Vermeulen S, Amalfitano A, Bachelot Y, Bouchard P, Cabrol S, Carel JC, Delemarre-van de Waal H, Goulet-Salmon B, Kottler ML, Richard O, Sanchez-Franco F, Saura R, Young J, Petit C, Hardelin JP. (2003). Loss-of-function mutations in FGFR1 cause autosomal dominant Kallmann syndrome. *Nature Genetics*. 33:463-465.
- Dodé C, Teixeira L, Levilliers J, Fouveaut C, Bouchard P, Kottler ML, Lespinasse J, Lienhardt-Roussie A, Mathieu M, Moerman A, Morgan G, Murat A, Toublanc JE, Wolczynski S, Delpech M, Petit C, Young J, Hardelin JP. (2006). Kallmann Syndrome: mutations in the genes encoding prokineticin-2 and prokineticin receptor-2. *Plos Genetics*. 2(10):1648-1652.
- Dodé C, Hardelin JP. (2010). Clinical genetics of Kallmann síndrome. *Annales d´endocrinologie*. 71(3):149-57.
- Dubois EA, Zandbergen MA, Peute J, Goos HJ. (2002). Evolutionary development of three GnRH systems in vertebrates. *Brain Research Bulletin.* 57: 413-418.
- Eaton and Glasgow (2007). Zebrafish orthopedia (otp) is required for isotocin cell development. *Development Genes and Evolution*. 217(2):149-58.

- Ebling FJ. (2005). The neuroendocrine timing of puberty. *Reproduction*. 129:675-683.
- Eisthen HL, Delay RJ, Wirsig-Wiechmann CR, Dionne VE. (2000). Neuromodulatory effects of gonadotropin releasing hormone on olfactory receptor neurons. *Journal of Neuroscience*. 20:3947-3955.
- El Amraoui A, Dubois PM (1993). Experimental evidence for an early commitment of gonadotropin-releasing hormone neurons, with special regard to their origin from the ectoderm of nasal cavity presumptive territory. *Neuroendocrinology*. 57(6): 991-1002.
- Fico A, Maina F, Dono R. (2011). Fine-tuning of cell signaling by glypicans. *Cellular and Molecular. Life Sciences.* 68:923-929.
- Fink G. (1988). Gonadotropin secretion and its control. *The physiology of reproduction*. Chapter 32.
- Forni PE, Taylor-Burds C, Senkus Melvin V, Williams T, Wray S. (2011). Neural Crest and Ectodermal Cells Intermix in the Nasal Placode to Give Rise to GnRH-1 Neurons, Sensory Neurons, and Olfactory Ensheathing Cells. *The Journal of Neuroscience*. 31(18):6915– 6927.
- Franz T. (1994). Extra-toes (Xt) homozygous mutant mice demonstrate a role for the Gli3 gene in the development of the forebrain. *Acta Anatomica.* 150:38-44.
- Fu M, Lui VCH, Sham MH, Pachnis V, Tam PKH. (2007). Sonic hedgehog regulates the proliferation, differentiation, and migration of enteric neural crest cells in gut. *The journal of cell biology*. 166(5): 673-684.
- Fukuchi-Shimogori T, Grove EA. (2003). Emx2 patterns the neocortex by regulating

FGF positional signaling. *Nature Neuroscience*. 6(8):825-31.

- Galli A, Roure A, Zeller R, Dono R. (2003). Glypican 4 modulates FGF signalling and regulates dorsoventral forebrain patterning in Xenopus embryos. *Development*. 130:4919-4929.
- Gong Q, Chen H, Farbman AI. (2009). Olfactory Sensory Axon Growth and Branching Is Influenced by Sonic Hedgehog. *Developmental Dynamics*. 238:1768-1776.
- González-Martínez D, Kim SH, Hu Y, Guimond S, Schofield J, Winyard P, Vannelli GB, Turnbull J and Bouloux PM (2004). Anosmin-1 Modulates Fibroblast Growth Factor Receptor 1 Signaling in Human Gonadotropin-Releasing Hormone Olfactory Neuroblasts through a Heparan Sulfate-Dependent Mechanism. *The Journal of Neuroscience*. 24(46):10384 –10392.
- Gopinath A, Tseng LA, Whitlock KE. (2004). Temporal and spatial expression of gonadotropin releasing hormone (GnRH) in the brain of developing zebrafish (Danio rerio). *Gene Expression Patterns*. 4:65–70.
- Guimond SE, Turnbull JE. (1999). Fibroblast growth factor receptor signaling is dictated by specific heparan sulphate saccharides. *Current Biology*. 9(22):1343-1346.
- Hallonet M, Hollemann T, Pieler T, Gruss P. (1999). *Vax1*, a novel homeobox containing gene, directs development of the basal forebrain and visual system. *Genes and Development*. 13:3106-3114.
- Han C, Belenkaya TY, Khodoun M, Tauchi M, Lin X. (2004). Drosophila glypicans control the cell-to-cell movement of Hedgehog by a dynamin-independent process.

Development. 131:601-611.

- Herzog W, Zeng X, Lele Z, Sonntag C, Ting JW, Chang CY, Hammerschmidt M. (2003). Adenohypophysis formation in the zebrafish and its dependence on Sonic hedgehog. *Developmental Biology*. 254:36-49.
- Hu Y, Tanriverdi F, MacColl GS, Bouloux PMG. (2003). Kallmann's syndrome: molecular pathogenesis. *International Journal of Biochemistry & Cell Biology*.35:1157–1162.
- Kallmann FJ, Schoenfeld WA, Barrera SE (1944). The genetic aspects of primary eunuchoidism. *American Journal of Mental Deficiency*. 48:203-236.
- Kalstrom RO, Trowe T, Klostermann S, Baier H, Brand M, Crawford AD, Grunewald B, Haffter P, Hoffmann H, Meyer SU, Müller BK, Richter S, van Eeden FJM, Nüsslein-Volhard C, Bonhoeffer F. (1996). Zebrafish mutations affecting retinotectal axon pathfinding. *Development*. 123:427-438.
- Kalstrom RO, Talbot WS, Schier AF. (1999). Comparative synteny cloning of zebrafish *you-too*: mutations in the hedgehog target *gli2* affect ventral forebrain patterning. *Genes and Development.* 13:388-393.
- Kaser A, Winklmayr M, Lepperdinger G, Kreil G. (2003). The AVIT protein family. Secreted cysteine-rich vertebrate proteins with diverse functions. *EMBO reports*. 4(5):469-473.
- Kawahara A, David IB. (2002). Developmental expression of zebrafish emx1 during early embryogenesis. *Gene Expression Patterns*. 2:201–206.
- Kawai T, Oka Y, Eisthen H. (2009). The role of the terminal nerve and GnRH in olfactory system neuromodulation. *Zoological Science*. 26:669-680.

- Kim HG, Layman LC. (2011).The role of CHD7 and the newly identified WDR11 gene in patients with idiopathic hypogonadotropic hypogonadism and Kallmann syndrome. *Molecular and cellular endocrinology*. 346:74-83.
- Kim HG, Ahn JW, Kurth I, Ullmann R, Kim HT, Kulharya A, Ha KS, Itokawa Y, Meliciani I, Wenzel W, Lee D, Rosenberger G, Ozata M, BickvDP, Sherins RJ, Nagase T, Tekin M, Kim SH, Kim CH, Ropers HH, Gusella JF, Kalscheuer V, Choi CY, Layman LC. (2010). WDR11, a WD Protein that Interacts with Transcription Factor EMX1, Is Mutated in Idiopathic Hypogonadotropic Hypogonadism and Kallmann Syndrome. *The American Journal of Human Genetics*. 87:465–479.
- Kim HK, Smith KM and Whitlock KE (2006). Disruption of kallmann and fgfr1 gene function in zebrafish differentially affects GnRH and olfactory cell development. XXVIII Annual Meeting of the Association for Chemical Senses, Sarasota Florida, USA. *Chemical Senses*. 31: A1-A144.
- Kim H, Maturana C, Letelier J, Whitlock K. (2011). Kallmann Syndrome is correlated with disrupted patterning of the hypothalamus. *Unpublish results*.
- Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF. (1995). Stages of Embryonic Development of the Zebrafish. *Developmental Dynamics*. 203:253-310.
- Krauss S, Concordet JP, Ingham PW. (1993). A functionally conserved homolog of the Drosophila segment polarity gene hh is expressed in tissues with polarizing activity in zebrafish embryos. *Cell*. 75(7):1431-44.
- Kuschel S, Ruther U, Theil T. (2003). A disrupted balance between Bmp/Wnt and Fgf signaling underlies the ventralization of the *Gli3* mutant telencephalon. *Developmental Biology*. 260:484–495.

- LeCouter J, Lin R, Tejada M, Frantz G, Peale F, Hillan KJ, Ferrara N. (2003). The endocrine-gland-derived VEGF homologue Bv8 promotes angiogenesis in the testis: Localization of Bv8 receptors to endothelial cells. *PNAS*. 100(5):2685–2690.
- Le Douarin NM, Kalchein C. (1999). The Neural Crest. *Cambridge University Press*.
- Leroy C, Fouveaut C, Leclercq S, Jacquemont S, Du Boullay H, Lespinasse J, Delpech M, Dupont JM, Hardelin JP, Dode C. (2008). Biallelic mutations in the prokineticin-2 gene in two sporadic cases of Kallmann syndrome. *European Journal of Human Genetics*. 16, 865–868.
- Li Y, Allende ML, Finkelstein R, Weinberg ES. (1994). Expression of two zebrafish *orthodenticle*-related genes in the zebrafish embryonic brain. *Mechanisms of Development*. 48:229-244.
- Li M, Bullock CM, Knauer DJ, Ehlert FJ, Zhou QY. (2001). Identification of two prokineticin cDNAs: recombinant proteins potently contract gastrointestinal smooth muscle. *Molecular Pharmacology*. 59:692-698.
- Lichtneckert R, Nobs Z, Reichert H. (2008). Empty spiracles is required for the development of olfactory projection neuron circuitry in Drosophila. *Development*. 135:2415–2424.
- Lin DCH, Bullock CM, Ehlert FJ, Chen JL, Tian H, Zhou QY. (2002). Identification and Molecular Characterization of Two Closely Related G Protein-coupled Receptors Activated by Prokineticins/Endocrine Gland Vascular Endothelial Growth Factor. *The Journal of Biological Chemistry*. 277(22):19276–19280
- Liu NA, Huang H, Yang Z, Herzog W, Hammerschmidt M, Lin S, Melmed S. (2003).

Pituitary corticotroph ontogeny and regulation in transgenic zebrafish. *Molecular Endocrinol.* 17:959-966.

- Luxardi G, Galli A, Forlani S, Lawson K, Maina F, Dono R. (2007). Glypicans are differentially expressed during patterning and neurogenesis of early mouse brain. *Biochemical and Biophysical Research Communications*. 352:55–60.
- MacColl G, Bouloux P, Quinton R. (2002). Kallmann syndrome: adhesion, afferents and anosmia. *Neuron*. 34:675-678.
- Marsango S, Bonaccorsi di Patti MC, Barra D, Miele R. (2010). Evidence that prokineticin receptor 2 exists as a dimer in vivo. *Cellular and Molecular Life Sciences*.
- Martin C, Balasubramanian R, Dwyer AA, Au MG, Sidis Y, Kaiser UB, Seminara SB, Pitteloud N, Zhou QY, Crowley Jr WF. (2011). The Role of the Prokineticin 2 Pathway in Human Reproduction: Evidence from the Study of Human and Murine Gene Mutations. *Endocrine Reviews*. 32(2):225-246.
- Mason AJ, Pitts SL, Nikolics K, Szonyi E, Wilcox JN, Seeburg PH, Stewart TA. (1986). The hypogonadal mouse: reproductive functions restored by gene therapy. *Science*. 234:1372–1378.
- Masuda Y, Takatsu Y, Terao Y, Kumano S, Ishibashi Y, Suenaga M, Abe M, Fukusumi S, Watanabe T, Shintani Y, Yamada T, Hinuma S, Inatomi N, Ohtaki T, Onda H, Fujino M. (2002). Isolation and identification of EG-VEGF/prokineticins as cognate ligands for two orphan G-protein-coupled receptors. *Biochemical and Biophysical Research Communications*. 293:396-402.
- Mathieu J, Barth A, Rosa FM, Wilson SW, Peyrieras N. (2002). Distinct and

cooperative roles for Nodal and Hedgehog signals during hypothalamic development. *Development*. 129:3055–3065.

- Matsumoto S, Yamazaki C, Masumoto K, Nagano M, Naito M, Soga T, Hiyama H, Matsumoto M, Takasaki J, Kamohara M, Matsuo A, Ishii H, Kobori M, Katoh M, Matsushime H, Furuichi K, Shigeyoshi Y. (2006). Abnormal development of the olfactory bulb and reproductive system in mice lacking prokineticin receptor PKR2. *PNAS*. 103(11): 4140-45.
- McCurley AT, Callard GV. (2008). Characterization of housekeeping genes in zebrafish: male-female differences and effects of tissue type, developmental stage and chemical treatment. *BMC Molecular Biology*. 9:102.
- Meyers EN, Lewandoski M, Martin GR. (1998). An Fgf8 mutant allelic series generated by Cre- and Flp-mediated recombination. *Nature Genetics*. 18:136-141.
- Meza-Vázquez HE, Serrano EA, Ramos F, Ramos AM, Sánchez FJ. (2011). Síndrome de Kallmann. Reporte de cuatro casos. *Boletín Colegio Mexicano de Urología*. XXVI(1): 35-38.
- Miyake A, Nakayama Y, Konishi M, Itoh N. (2005). Fgf19 regulated by Hh signaling is required for zebrafish forebrain development. *Developmental Biology*. 288:259-275.
- Miyasaka N, Sato Y, Yeo SY, Hutson LD, Chien CB, Okamoto H, Yoshihara Y. (2005). Robo2 is required for establishment of a precise glomerular map in the zebrafish olfactory system. *Development*. 132:1283-1293.
- Morita T, Nitta H, Kiyama Y, Mori H, Mishina M. (1995). Differential expression of two zebrafish emx homeoprotein mRNAs in the developing brain. *Neuroscience*

Letters. 198: 131-134.

- Nasevicius A, Ekker SC. (2000). Effective targeted gene “knockdown” in zebrafish. *Nature Genetics*. 26: 216-220.
- Negri L, Lattanzi R, Giannini E, Melchiorri P. (2007). Bv8/Prokineticin proteins and their receptors. *Life Sciences*. 81:1103-1116.
- Ng KL, Li JD, Cheng MY, Leslie FM, Lee AG, Zhou QY. (2005). Dependence of Olfactory Bulb Neurogenesis on Prokineticin 2 Signaling. *Science*. 308:1923-27.
- Ngan ES, Shum CK, Poon HC, Sham MH, Garcia-Barceló MM, Lui VC, Tam PK. (2008). Prokineticin-1 (Prok-1) works coordinately with glial cell line-derived neurotrophic factor (GDNF) in enteric neural crest cells. *BBA Molecular Cell Research*. 1783(3): 467-478.
- Ngan ES, Lee KY, Sit FY, Poon HC, Chan JK, Sham MH, Lui VC, Tam PK. (2007). Prokineticin-1 modulates proliferation and differentiation of enteric neural crest cells. *BBA Molecular Cell Research*. 1773(4): 536-545.
- Ngan ESW, Tam PKH. (2008). Prokineticin-signaling pathway. *The International Journal of Biochemistry & Cell Biology*. 40:1679-1684.
- Okada T, Okumura Y, Motoyama J, Ogawa M. (2008). FGF8 signaling patterns the telencephalic midline by regulating putative key factors of midline development. *Developmental Biology*. 320:92-101.
- Ohkubo Y, Chiang C, Rubenstein JL. (2002). Coordinate regulation and synergistic actions of BMP4, SHH and FGF8 in the rostral prosencephalon regulate morphogenesis of the telencephalic and optic vesicles. *Neuroscience*. 111:1-17.
- Okubo K, Sakai F, Lau EL, Yoshizaki G, Takeuchi Y, Naruse K, Aida K, Nagahama

Y. (2006). Forebrain GnRH neuronal development: insights from transgenic medaka and the relevance to X-linked KS. *Endocrinology*.147(3):1076-1084.

- O'Leary DD, Nakagawa Y (2002). Patterning centers, regulatory genes and extrinsic mechanisms controlling arealization of the neocortex. *Current Opinion in Neurobiology*. 12:14-25.
- Parhar IS, Soga T, Ishikawa Y, Nagahama Y, Sakuma Y. (1998). Neurons synthesizing GnRH mRNA subtypes have multiple developmental origins in the medaka. *The Journal of comparative Neurology*. 401:217-226.
- Park MK, Wakabayashi K. (1986). Preparation of a monoclonal antibody to common amino acid sequence of LHRH and its application. *Endocrinol Jpn*. 33:257-272.
- Peng Z, Tang Y, Luo H, Jiang F, Yang J, Sun L, Li JD. (2011). Disease-causing Mutation in PKR2 Receptor Reveals a Critical Role of Positive Charges in the Second Intracellular Loop for G-protein Coupling and Receptor Trafficking. *The Journal of Biological Chemistry*. 286(19):16615-16622.
- Pitteloud N, Zhang C, Pignatelli C, Li JD, Raivio T, Cole LW, Plummer L, Jacobson-Dickman EE, Mellon PL, Zhou QY, Crowley Jr WF. (2007). Loss-of-function mutation in the prokineticin 2 gene causes Kallmann syndrome and normosmic idiopathic hypogonadotropic hypogonadism. *PNAS*. 104(44):17447-52.
- Prosser HM, Bradley A, Caldwell MA. (2007). Olfactory bulb hypoplasia in Prokr2 null mice stems from defective neuronal progenitor migration and differentiation. *European Journal of Neuroscience*. 26:3339–3344.
- Puverel S, Nakatani H, Parras C, Soussi-Yanicostas N. (2009). Prokineticin

Receptor 2 Expression Identifies Migrating Neuroblasts and Their Subventricular Zone Transient-Amplifying Progenitors in Adult Mice. *The Journal of Comparative Neurology*. 512:232–242.

- Quinton R, Duke VM, Robertson A, Kirk JMW, Matfin G, de Zoysa PA, Azcona C, MacColl GS, Jacobs HS, Conway GS, Besser M, Stanhope RG, Bouloux PM. (2001). Idiopathic gonadotrophin deficiency: genetic questions addressed through phenotypic characterization. *Clinical Endocrinology*. 55:163-174.
- Rasband WS, ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/, 1997-2009.
- Reichenbach B, Delalande JM, Kolmogorova E, Prier A, Nguyen T, Smith CM, Holzschuh J, Shepherd IT. (2008). Endoderm-derived Sonic hedgehog and mesoderm Hand2 expression are required for enteric nervous system development in zebrafish. *Developmental Biology*. 318:52–64
- Ribes V, Wang Z, Dollé P, Niederreither K. (2006) Retinaldehyde dehydrogenase 2 (RALDH2)- mediated retinoic acid synthesis regulates early mouse embryonic forebrain development by controlling FGF and sonic hedgehog signaling. *Development*. 133:351-361.
- Rozen S, Skaletsky H. (2000). Primer3 on the WWW for general users and for biologist programmers. In: Krawetz S, Misener S (eds). *Bioinformatics methods and protocols: Methods in molecular biology*. Humana Press, Totowa, NJ, pp 365-386.
- Rugarli EI, Ghezzi C, Valsecchi V, Ballabio A. (1996). The Kallmann syndrome gene product expressed in COS cells is cleaved on the cell surface to yield a diffusible component. *Human Molecular Genetics*. 5(8):1109–1115.

- Sarnat HB, Flores-Sarnat L. (2001). Neuropathologic research strategies in holoprosencephaly. *Journal of Child Neurology*. 16:918-931.
- Schneider RA, Hu D, Rubenstein JL, Maden M, Helms JA. (2001) Local retinoid signaling coordinates forebrain and facial morphogenesis by maintaining FGF8 and SHH. *Development*. 128:2755-2767.
- Scholpp S, Groth C, Lohs C, Lardelli M, Brand M. (2004). Zebrafish fgfr1 is a member of the fgf8 synexpression group and is required for fgf8 signaling at the midbrain-hindbrain boundary. *Development Genes and Evolution*. 214:285-295.
- Schwandel-Fukuda M, Pfaff DW. (1989). Origin of luteinizing hormone-releasing hormone neurons. *Nature*. 338:161-163.
- Shanmugalingam S, Houart C, Picker A, Reifers F, Macdonald R, Barth A, Griffin K, Brand M, Wilson SW. (2000). Ace/Fgf8 is required for forebrain commissure formation and patterning of the telencephalon. *Development*. 127: 2549–2561.
- Sisk CL, Foster DL. (2004). The neural basis of puberty and adolescence. *Nature Neuroscience*. 7:1040:1047.
- Soga T, Matsumoto S, Oda T, Saito T, Hiyama H, Takasaki J, Kamohara M, Ohishi T, Matsushime H, Furuichi K. (2002). Molecular cloning and characterization of prokineticin receptors. *Biochimica et Biophysica Acta*. 1579:173– 179.
- Soga T, Ogawa S, Millar RP, Sakuma Y, Parhar IS. (2005). Localization of the three GnRH types and GnRH receptors in the brain of a cichlid fish: insights into their neuroendocrine and neuromodulator functions. *The journal of comparative neurology*. 487:28-41.
- Soussi-Yanicostas N, Hardelin JP, Arroyo-Jimenez M, Ardouin O, Legouis R,

Levilliers J, Traincard F, Betton JM, Cabanié L, Petit C. (1996). Initial characterization of anosmin-1, a putative extracellular matrix protein synthesized by definite neuronal cell populations in the central nervous system. *Journal of Cell Science*. 109:1749-1757.

- Stell WK, Walker SE, Ball AK. (1987). Functional-anatomical studies on the Terminal nerve projection to the retina of bony fishes. *Annals of the New York Academy of Science*. 519:80-96.
- Sykiotis GP, Hoang XH, Avbelj M, Hayes FJ, Thambundit A, Dwyer A, Au M, Plummer L, Crowley WF Jr., Pitteloud N. (2010). Congenital Idiopathic Hypogonadotropic Hypogonadism: Evidence of Defects in the Hypothalamus, Pituitary, and Testes. *Journal of Clinical Endocrinology and Metabolism*. 95(6):3019–3027.
- Takahashi H, Liu FC. (2006). Genetic Patterning of the Mammalian Telencephalon by Morphogenetic Molecules and Transcription Factors. *Birth Defects Research* (Part C). 78:256–266.
- Theil T, Alvarez-Bolado G, Walter A, Ruther U. (1999). Gli3 is required for Emx gene expression during dorsal telencephalon development. *Development*. 126: 3561-3571.
- Theil T, Aydin S, Koch S, Grotewold L, Rüther U. (2002). Wnt and Bmp signalling cooperatively regulate graded Emx2 expression in the dorsal telencephalon. *Development*. 129:3045-3054.
- Thisse C, Thisse B, Schilling TF, Postlethwait JH. (1993). Structure of the zebrafish *snail1* gene and its expression in wild-type, spadetail and no tail mutant embryos.

Development. 119:1203-1215.

- Tole S, Ragsdale CW, Grove EA. (2000). Dorsoventral patterning of the telencephalon is disrupted in the mouse mutant extra-toes. *Developmental Biology.* 217:254–265.
- Tornberg J, Sykiotis GP, Keefe K, Plummer L, Hoang X, Hall JE, Quinton R, Seminara SB, Hughes V, Vliet GV, Uum SV, Crowley WF, Habuchi H, Kimata K, Pitteloud N, Bülow HE. (2011). Heparan sulfate 6-O-sulfotransferase 1, a gene envolved in extracellular sugar modifications, is mutated in patients with idiopathic hypogonadotrophic hypogonadism. *PNAS*. 108(28):11524-11529.
- Treier M, O'Connell S, Gleiberman A, Price J, Szeto DP, Burgess R, Chuang PT, McMahon AP, Rosenfeld MG. (2001). Hedgehog signaling is required for pituitary gland development. *Development*.128:377-386.
- Tsai PS, Gill J. (2006). Mechanism of disease: insights into X-linked and autosomal-dominant KS. *Nature Clinical Practice Endocrinology and Metabolism*. 2:160-171.
- Unger JL, Glasgow E. (2003). Expression of isotocin-neurophysin mRNA in developing zebrafish. *Gene expression patterns*. 3:105-108.
- Vitebsky A, Reyes R, Sanderson MJ, Michel WC and Whitlock KE. (2005). Isolation and Characterization of the *laure* Olfactory Behavioral Mutant in the Zebrafish, *Danio rerio*. *Developmental dynamics*. 234:229–242.
- Walshe J, Mason I. (2003). Unique and combinatorial functions of Fgf3 and Fgf8 during zebrafish forebrain development. *Development*. 130:4337-4349.
- Wang W, Lufkin T. (2000). The Murine *Otp* Homeobox Gene Plays an Essential

Role in the Specification of Neuronal Cell Lineages in the Developing Hypothalamus. *Developmental Biology*. 227:432–449.

- Westerfield, M. (1993). The Zebrafish Book: A Guide for the Laboratory Use of Zebrafish (Brachydanio rerio). *University of Oregon Press*.
- Whitlock KE, Westerfield M. (1998). A Transient Population of Neurons Pioneers the Olfactory Pathway in the Zebrafish. *The Journal of Neuroscience*. 18(21):8919–8927.
- Whitlock KE, Westerfield M. (2000). The olfactory placode of the zebrafish form by convergence of cellular fields at the edge of the neural plate. *Development.* 127: 3645-3653.
- Whitlock KE, Wolf CD, Boyce ML. (2003). Gonadotropin-releasing hormone (GnRH) cells arise from cranial neural crest and adenohypophyseal regions of the neural plate in the zebrafish, *Danio rerio*. *Dev Biol*. 257(1): 140-52.2.
- Whitlock KE. (2004). A new model for olfactory placode development. *Brain, Behavior and Evolution.* 64: 126-140.
- Whitlock KE, Smith KM, Kim H, Harden MV. (2005a). A role for *foxd3* and *sox10* in the differentiation of gonadotropin-releasing hormone (GnRH) cells in the zebrafish Danio rerio. *Development*. 132(24):5491-502.
- Whitlock KE. (2005b). Origin and development of GnRH neurons. *Trends in Endocrinology and Metabolism.* 16(4):145-151.
- Whitlock KE. (2006). Development of GnRH cells: setting the stage for puberty. *Molecular and Cellular Endocrinology.* 254-55:39-50.
- Wirsig-Wiechmann CR, Wiechmann AF, Eisthen HL. (2002). What defines the

nervus terminalis? Neurochemical, developmental, and anatomical criteria. *Progress in brain research*. 141:45-58

- Witkin JW, Dao D, Livne I, Dunn IC, Zhou XL, Pula K, Silverman AJ. (2003). Early expression of chicken GnRH1 in the developing chick. *Journal of Neuroendocrinology*. 15:865-870.
- Wray S, Grant P, Gainer H. (1989a). Evidence that cells expressing luteinizing hormone-releasing hormone mRNA in the mouse are derived from progenitor cells in the olfactory placode. *PNAS USA*. 86:8132-8136.
- Wray S, Nieburgs A, Elkabes S. (1989b). Spatiotemporal cell expression of luteinizing hormone-releasing hormone in the prenatal mouse: Evidence for an embryonic origin in the olfactory placode. *Brain Research Dev Brain Research*. 46:309-318.
- Wullimann MF, Rupp B, Reichert H. (1996). Neuroanatomy of the Zebrafish Brain: a Topological Atlas. *Birkhäuser Verlag: Basel*. 1-144.
- Yan D and Lin X. (2009). Shaping Morphogen Gradients by Proteoglycans. *Cold Spring Harb Perspect Biol*. 1:a002493.
- Yanicostas C, Herbomela E, Dipietromariaa A, Soussi-Yanicostas N. (2009). Anosmin-1a is required for fasciculation and terminal targeting of olfactory sensory neuron axons in the zebrafish olfactory system. *Molecular and Cellular Endocrinology*. 312:53-60.
- Yoshida M, Suda Y, Matsuo I, Miyamoto N, Takeda N, Kuratani S, Aizawa S (1997). Emx1 and Emx2 functions in development of dorsal telencephalon. *Development*. 124:101-111.

- Zembrzycki A, Griesel G, Stoykova A, Mansouri A. (2007). Genetic interplay between the transcription factors Sp8 and Emx2 in the patterning of the forebrain. *Neural Development* 2007:2(8).
- Zhang F, Zhang Z, Lin X, Beenken A, Eliseenkova AV, Mohammadi M, Linhardt RJ. (2009). Compositional Analysis of Heparin/Heparan Sulfate Interacting with Fibroblast Growth Factor 3 Fibroblast Growth Factor Receptor Complexes. *Biochemistry*. 48:8379-8386.
- Zhou QY. (2006). The Prokineticins, a novel pair of regulatory peptides. *Molecular interventions*. 6(6):330-38.