Knocking-down of the Prokineticin receptor 2 affects reveals its complex role in the regulation of the hypothalamus-pituitary-gonadal axis in the zebrafish model

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Prokineticin receptors (PROKR1 and PROKR2) are G protein-coupled receptors which control human central and peripheral reproductive processes. Importantly, allelic variants of PROKR2 in humans are associated with altered migration of GnRH neurons, resulting in congenital hypogonadotropic hypogonadism (CHH), a heterogeneous disease characterized by delayed/absent puberty and/or infertility. Although this association is established in humans, murine models failed to fully recapitulate the reproductive and olfactory phenotypes observed in patients harboring PROKR2 mutations. Here, taking advantage of zebrafish model we investigated the role of prokr1b (ortholog of human PROKR2) during early stages of GnRH neuronal migration. Real-Time PCR and whole mount in situ hybridization assays indicate that prokr1b spatial-temporal expression is consistent with gnrh3. Moreover, knockdown and knockout of prokr1b altered the correct development of GnRH3 fibers, a phenotype that is rescued by injection of prokr1b mRNA. These results suggest that prokr1b regulates the development of the GnRH3 system in zebrafish. Analysis of gonads development and mating experiments indicate that prokr1b is not required for fertility in zebrafish, although its loss determine changes also at the testis level. Altogether, our results support the thesis of a divergent evolution in the control of vertebrate reproduction and provide a useful in vivo model for deciphering the mechanisms underlying the effect of PROKR2 allelic variants on CHH.

The hypothalamus-pituitary-gonadal (HPG) axis controls reproduction in vertebrates1-5 through the pulsatile release of gonadotropin-releasing hormone (GnRH) hormone by GnRH neurons. During development, these neurons differentiate from neural crest cells and ectodermal progenitors in a niche at the border between the respiratory epithelium and the vomeronasal/olfactory epithelium. From these regions these neurons migrate caudally to reach the medio-basal hypothalamus, where they complete their differentiation6. Several studies in different animal models have shown that specific evolutionarily conserved genes regulate this migratory process and the functions of these neurons. The role of these genes in human fertility is supported by the existence of variants in these genes associated with congenital hypogonadotropic hypogonadism (CHH)7, a rare and clinically heterogeneous disorder characterized by abnormal pubertal development and/or infertility, a normal (nCHH) or defective sense of smell (Kallmann syndrome, KS) and other reproductive and non-reproductive anomalies8-10. More than 25 genes have been associated with CHH, although variants in these genes account only for 40–50% of reported cases. Several evidences indicate that CHH is a complex genetic disease characterized by variable expressivity and penetrance of the associated genetic defects, which can be partially explained by an oligogenic-inheritance model9,11. Among the genes involved in CHH, prokineticin receptor 2 (PROKR2) has an important role.

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in GnRH neuron migration. PROKR2, as a member of the GPCR family, has an extracellular amino-terminal end, an intracellular carboxy-terminal domain and a central core formed by seven transmembrane α-helical segments (TM1–TM7)[12]. Prokineticins have been previously demonstrated to be involved in several physiological functions in neurogenesis, regulation of circadian rhythms, metabolism, angiogenesis, pain perception, muscle contractility, hematopoiesis, immune response, thermoregulation and energy expenditure[13–15]. Matsumoto and colleagues in 2006 reported the first observation that Prokr2 knock-out mice display hypoplastic gonads and olfactory structures, a phenotype reminiscent of KS, to link this gene to CHH[16]. Furthermore, Prokr2 knock-out mice show decreased plasma levels of testosterone and follicle-stimulating hormone, but not luteinizing hormone. In humans, genetic screening of CHH cohorts has revealed mutations in PROKR2 in KS and nCHH patients, but mostly in the heterozygous state[17–20]. Moreover, studies on transfected cells demonstrated that the missense PROKR2 variants observed in KS and nCHH patients have a deleterious effect on PROKR2 signaling[18,20,21], although such variants are also present in apparently unaffected individuals[22–24]. Thus, despite several in vitro and in vivo studies, the role of Prokr2 in GnRH neuron function and in CHH pathogenesis remains incompletely understood. Here, we take advantage of the zebrafish to generate an in vivo model to investigate PROKR2 function during GnRH neuron ontogeny. Using both transient knockdown and germline knockout of prokr1b, the zebrafish ortholog of human PROKR2, we find that prokr1b has an important role in the migration of GnRH axons, but not for fertility, in this animal model.

**Results**

Mammals possess two prokineticin receptors named PROKR1 and PROKR2[25]. The zebrafish genome contains two prokr paralogs named prokr1a and prokr1b (or prokr1l), and previous evidence suggests that prokr1a and prokr1b correspond to mammalian PROKR1 and PROKR2, respectively[26]. To determine which of these genes may be involved in GnRH neuronal migration, we evaluated their expression during zebrafish embryo development. Real-Time PCR analysis on RNA extracts from whole embryos revealed that prokr1b is expressed at higher levels compared to prokr1a and exhibits an increase from 24 hours post-fertilization (hpf) to 72 hpf (Fig. 1A,B).

Whole-mount in situ hybridization (WISH) performed at the same developmental stages (Fig. 2) revealed that prokr1b, but not prokr1a, is expressed in cells adjacent to the olfactory placodes (Fig. 2G-L), similar to the pattern observed for gnrh3 (Fig. 2M–R). Given the role of PROKR2 in the migration of GnRH neurons in humans, these results suggest that prokr1b may similarly be involved in GnRH3 neuron development in zebrafish.

Next we used morpholino anti-sense oligonucleotides (MOs) to knock-down expression of prokr1a or prokr1b in tg (gnrh3:EGFP) embryos, which express EGFP in GnRH3 cells[27] (Supplementary Fig. S1A–C). Images collected at 48 hpf (Fig. 3) revealed that prokr1a knock-down (Fig. 3C) did not affect the development of GnRH3 fibers that appeared similar to those of uninjected wild-type animals (WT, Fig. 3A) or injected with a control MO (ctrl-MO) (Fig. 3B). In contrast, knock-down of prokr1b led to evident alterations in the architecture of the GnRH3 network (Fig. 3D). In these embryos, GnRH3 fibers appeared disorganized, especially at the level of the anterior commissure (AC) and in the anterior fibers (dotted square in Fig. 3D; Supplementary Fig. S1D). These results suggest that prokr1b is required for normal GnRH neuron development in zebrafish.

To confirm the defects observed for prokr1b knock-down using MOs, a technique that is prone to non-specific artefacts, we next analyzed GnRH3 neuron development in animals containing a germline mutation in prokr1b[28]. To do so, we compared EGFP-expressing GnRH3 neuron fibers in prokr1b homozygous mutant, heterozygous mutant, and homozygous WT siblings at 48 hpf and 72 hpf. No differences were observed between prokr1b<sup>−/−</sup> and WT siblings at these two time points (Fig. 4A,B,E,F), while prokr1b<sup>−/−</sup> embryos (Fig. 4C,G) showed defects in GnRH3 neuron fibers in the same anatomical region as in the knockdown experiments (Fig. 3D). This phenotype appears to be specific to the absence of prokr1b, as injection of WT prokr1b, but not prokr1a mRNA into prokr1b<sup>−/−</sup> animals at the 1-cell stage rescued the phenotype (Fig. 4D–H; Supplementary Fig. S2E,F).

These observations were supported by quantitative analysis of GnRH3 neuron fibers (Fig. 5A,B), providing an evidence for a role of prokr1b in GnRH neuron development in zebrafish.

In order to establish whether prokr1b is required for the development and function of the reproductive system in zebrafish, as it is in humans and mice, we compared the reproductive organs and fecundity of prokr1b<sup>−/−</sup>,
prokr1a probe (A–F) did not display any signal in the head of zebrafish embryos. In contrast, prokr1b (G–L) is expressed in cells near the olfactory region at 36 hpf (G,H), 48 hpf (I,J) and 72 hpf (K,L). That is similar to gnrh3 expression at these times (M–R). Panels A,C,E,G,I,K,M,O and Q show lateral views. Panels B,D,F,H,J,L,N,P and R show dorsal views of zebrafish embryos. Scale bar = 50 µm. Image taken using Leica Application Suite software (LAS version 4.7.0; https://www.leica-microsystems.com/products/microscope-software/p/leica-application-suite/).

Figure 3. Injection of ctrl-MO (B) or prokr1a-MO (C) does not affect GnRH3 neuron fibers, as they look comparable to uninjected WT (A) embryos. In contrast, knockdown of prokr1b (D) causes alteration in axons migration of the rostral part (dotted square in Fig A) and at the level of the anterior commissure (AC) of the embryos. OB = olfactory bulbs, OC = optic chiasm, AC = anterior commissure. Scale bar = 50 µm.

prokr1+/− and prokr1b+/−/+ siblings. Histological sections of testes and ovaries of 3-months-old male and female animals did not reveal obvious differences among the three genotypes, suggesting that prokr1b is not required for gonadal maturation in both sexes and, by consequence, for puberty in zebrafish (Fig. 6A–L). No differences were also observed among the three genotypes in the number of fertile eggs generated (Fig. 6M), neither in the GSI index of mutated male or female compared to WT indicating that prokr1b is not fundamental for fertility in zebrafish (Fig. 6N). Nevertheless, Real-Time qPCR conducted on tissue of adult fish, revealed for mutated males higher expression of lhrβ and fshrβ in the brain (Fig. 6O) together with a strong decrease of the gonadotropic receptors lhr and fshr in the gonads (Fig. 6P).
Discussion
Several recent studies have demonstrated a remarkable evolutionary conservation of the developmental migration of GnRH neurons and of several genes involved in GnRH ontogeny. In zebrafish, like in mammals, GnRH secreting neurons start their development at 24 hpf from cells located in the olfactory epithelium that send dorsal extensions that ultimately innervate the hypothalamus and pituitary. An important gene involved in the development of the GnRH system in humans is PROKR2. The zebrafish genome contains two prokineticin receptor paralogues, prokr1a and prokr1b. WISH analyses reveal that prokr1b expression starts in the brain at 24 hpf. The prokr1b mutant phenotype is rescued by injection with WT prokr1b mRNA.

Figure 4. prokr1b homozygous mutant zebrafish (tg(gnrh3:EGFP);prokr1b−/− (C,G) exhibit misrouting of rostral (dotted square) and optic chiasm (OC) fibers. These defects are not present in WT (A,E) and prokr1b heterozygous mutants (tg(gnrh3:EGFP);prokr1b+/− (B,F). The prokr1b mutant phenotype is rescued by injection with WT prokr1b mRNA (D,H). OB = olfactory bulbs, OC = optic chiasm, AC = anterior commissure. Scale bar = 50 µm.
ilar to humans with PROKR2 prokr1b of human, knockdown of gnrh3 implicated in the stimulation of the pituitary as previously suggested32,34,35.

and fshr expression levels in our male knockout fish, seem to confirm this stimulation, nevertheless they are not 

Figure 5. Quantification of GnRH3 fiber network confirms that prokr1b−/− embryos have reduced GnRH3 neuron projections, and that this phenotype is rescued by injection of prokr1b mRNA when analyzed at 48 hpf (A) or 72 hpf (B). For each group n = 10 animals. ns: not significant, ***p < 0.01.

although prokr1b appears to be the zebrafish ortholog of human and murine PROKR2, the zebrafish prokr1b mutant phenotype does not fully recapitulate the clinical features of CHH. Indeed, prokr1b mutation does not affect gonadal maturation or fertility in zebrafish, as demonstrated by fecundity testing and histological analysis of testis and ovaries at 3 months of age. Moreover, dorsoventral projections of GnRH3 neurons, despite reduced, are present in zebrafish prokr1b mutants at 72 hpf, in contrast to murine Prokr2 mutants in which there is an early arrest in GnRH neuronal migration36. Two recent studies conducted in zebrafish have highlighted differences in the role of the GnRH system during puberty and fertility. Lui and colleagues showed that triple mutants lacking gnrh3 and the 2 kisspeptin ligands undergo normal puberty and gonad maturation37. These results are surprising, because GnRH3 has been considered the most important stimulator of gonadotropin release in fish and its expression, together with kiss1 and kiss2, have been found to be higher during puberty and gonadal maturation in zebrafish38. Moreover Marvel and colleagues demonstrated that zebrafish gnrh2/gnrh3 double mutants show normal fertility, demonstrating that neither Gnrh2 nor kiss1 and kiss2, compensate for loss of GnRH3 in zebrafish39. Nevertheless, comparison between WT and double or triple mutants revealed in both studies different expression patterns of neuropeptides known to be important in mammal control of reproduction, such as tachykinin 3, secretogranin II and neuropeptide Y. These results suggest that, in contrast with mammals, multiple factors act in parallel with GnRH to stimulate the reproductive axis in zebrafish32,34,35. Our results in the knockout male fish might further confirm this hypothesis. Indeed, we reported a lhr and fshr lower expression in the testis that could firstly indicate a role of prokr1b in this organ similar to what already observed in mice, where absence of Prokr2 lead to a variable degree of compromised vasculature, even in the absence of evident structural gonadal modification36. Moreover, this could also be related to primary testes defect that has been described, in association to those in the hypothalamus and pituitary, also in human male with CHH (Sykiotis et al. JCEM 2010). Secondly, this reduced receptor expression might lead to a relative resistance to Lh and Fsh action which, in turn, might activates, through the negative feedback mechanism, the central compartment of the HPG axis. Higher lhβ and fshβ expression levels in our male knockout fish, seem to confirm this stimulation, nevertheless they are not consequence of level modification in gnrh3 expression levels. Thus, other factors from GnRH3 system might be implicated in the stimulation of the pituitary as previously suggested32,34,35.

In conclusion, even if mechanisms controlling the HPG and, by consequence, fertility have slightly diverged along evolution, these studies together demonstrate that genes regulating GnRH ontogeny present a certain degree of conservation among humans, mice and zebrafish32,34. Indeed, despite the variable phenotypic features, the Tg(gnrh3:EGFP);prokr1bcre;+/+ line presented here suggests that prokr1b is the orthologue of human PROKR2, and demonstrates that its loss affects the development of GnRH neuronal fibers in zebrafish, as in humans, but also the expression of the lhr and fshr at the testes level, thus indicating a complex implication of the prokinetin pathway in the HPG functionality. Moreover, this mutant lines a useful in vivo tool that, combined with mutant lines for other GnRH related genes, could contribute to our understanding of the development of the GnRH system and the complex mechanisms underlying CHH and related diseases.
Methods
Zebrafish lines and maintenance. Zebrafish (Danio rerio) embryos obtained from natural spawning were raised and maintained according to established techniques39. All experiments with live animals were performed at the University of Milan. All experimental protocols and methods were carried out in accordance with relevant
guidelines and regulations of Good Animal Practice approved by the institutional and licensing committee IACUC (Institutional Animal Care and Use Committee) and University of Milan by the Italian Decree of March 4th, 2014, n.26. Embryos were staged according to morphological criteria\(^\text{40}\). Beginning from 24 hpf, embryos were cultured in fish water containing 0.003% PTU (1-phenyl 2-thiourea; Sigma; Aldrich, Saint Louis, MO) to prevent pigmentation and 0.01% methylene blue to prevent fungal growth\(^\text{39}\). Wild-type (WT) zebrafish of the AB strain were obtained from the Wilson lab (University College London, London, United Kingdom). The tg (gnrh3:EGFP)\(^\text{27}\) and prokr1b\(^{ct814/c814}\)\(^\text{28}\) zebrafish lines have been previously described.

**Real-Time PCR.** Reverse Transcription-Polymerase Chain Reaction (RT-PCR) was performed on total RNA prepared from 20 zebrafish oocytes and embryos for each different developmental stages using the Total RNA Isolation Kit (Ambion, Thermo Fisher, Waltham MA) or the RNAagents Total RNA Isolation System (Promega, Madison, WI), treated with DNase I RNase free (Roche, Basel, Switzerland) to avoid possible contamination from genomic DNA. RNA concentrations and quality were determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, USA). Total RNA (1 µg) was reverse transcribed to produce cDNA using Superscript III reverse transcriptase (Invitrogen) primed with random hexamers, as described previously\(^\text{41}\). In all cases, a reverse transcriptase negative control was used to test for genomic DNA contamination. The primers used for quantitative Real-Time PCR are listed in the Supplementary Table S1.

**In situ hybridization.** Whole-mount in situ hybridization (WISH) was performed as described\(^\text{42}\). PCR products were cloned into the pGEM-T Easy vector (Promega, Table S2). The cDNA-containing plasmids were line- and transcribed with T7 and SP6 RNA polymerase (Roche) for antisense and sense riboprobe synthesis. Images of stained embryos were taken with a Leica MZFLIII epifluorescence stereomicroscope equipped with a DFC 480-R2 digital camera.

**Knockdown experiments.** We tested one antisense morpholino oligonucleotide (MO) each for prokr1a and prokr1b (Supplementary Table S3). Both were splice-blocking MOs synthesised by Gene Tools LLC (Oregon, USA). Morpholinos were dissolved in Danieau's solution (58 mMNaCl; 0.7 mM KCl; 0.4 mM MgSO\(_4\); H\(_2\)O; 0.6 mM Ca(NO\(_3\))\(_2\); 5 mM HEPES pH 7.2) at 2 mM and stored at \(-80^\circ\)C. Embryos were microinjected at the 1–4 cell stage with rhodamine dextran (Molecular Probes) co-injected as a tracer. As a control for non-specific effects, a standard control morpholino (ctrl-MO) was injected, which targets the human β-globin gene. Morpholinos were tested for efficacy and toxicity by injecting different doses in tg(gnrh3:EGFP) embryos and evaluating them for morphological defects (Supplementary Fig. S1A-C). After injection, embryos were raised in fish water at 28°C and observed until the developmental stage of interest. Embryos that were to be imaged after 24 hpf were treated with PTU. For imaging, embryos were anaesthetized using tricaine (ethyl 3-aminobenzoate methanesulfonate salt, Sigma; 25x stock = 0.08 g in 20 ml of distilled H\(_2\)O) in fish water. Injected embryos (morphants) were embedded at 48 hpf in UltraPure Low Melting Point Agarose (Thermo Fisher Scientific) and photographed using a confocal laser scanning microscope (Nikon C2) with a 20x objective.

**Generation of tg(gnrh3:EGFP); prokr1b\(^{ct814/c814}\) line and rescue experiments.** We crossed tg(gnrh3:EGFP); prokr1b\(^{ct814/c814}\) animals to prokr1f\(^{ct814/c814}\) animals\(^\text{38}\) to generate the tg(gnrh3:EGFP); prokr1f\(^{ct814/c814}\) line. Flip clipping was performed to isolate genetic material from individual fish for genotyping accordingly to what previous published in Chen and colleague\(^\text{26}\) (Table S4). The prokr1f\(^{ct814/c814}\) fish contain a 1 bp deletion (nucleotide 12 of the open reading frame: 5’-C-3’), which results in a change in reading frame after amino acid 4 and a premature stop codon after amino acid 13 compared to 396 amino acids for the wild-type (WT) protein. Rescue experiments were performed by injecting 400 pg prokr1b mRNA diluted in the Danieau's solution into 1-cell stage embryos.

**Live-imaging of GnRH3 fibers in prokr1b KO embryos.** To assess the role of prokr1b during GnRH3 fiber development, prokr1b KO animals were embedded at 48 or 72 hpf in UltraPure Low Melting Point Agarose (Thermo Fisher Scientific) and analysed using a confocal laser scanning microscope (Nikon C2+) with a 20x objective. GnRH3 fiber structure was assessed and 3D reconstructed using Fiji\(^\text{43}\). Due to the complexity of GnRH3 fibers, a specific region of interest (ROI) was selected and analyzed at each developmental stage (Supplementary Fig. S2), with background fluorescence subtracted from each image (Supplementary Fig. S2B–D). The number of green pixels within each ROI was used as a proxy for the amount of GnRH3 fibers.

**Gonads histology, fecundity/fertilization rates and GSI.** For histological analysis, gonads from 3-months-old fish were fixed in 4% paraformaldehyde (PFA), dehydrated, wax-embedded, cut into 8 µm sections using a microtome (Leitz 1516), and stained with eosin. Samples were imaged using a Leica DM6000 B microscope equipped with a Leica 480 digital camera using the Leica Application Suite (LAS version 4.7.0). The assessment of fecundity (number of eggs released) and fertilization rate (fraction of eggs that developed into an embryo), WT and mutant females and males at 3 months-old were paired in a spawning tray. After one hour, eggs were collected in 30% Danieau’s solution and counted. The number of fertilized and unfertilized eggs was discerned using a dissecting microscope at 6 hpf. Twelve-months-old male and female fish were then dissected to collect ovaries and testicles for gonadosomatic index (GSI) measurement and Real-time PCR. The GSI was calculated according to the formula (organosomatic index = organ weight × 100/body weight)\(^\text{44}\).

**Statistical analysis.** Statistical analyses in Fig. 5 was performed using one-way ANOVA with Dunnett’s post-hoc test using GraphPad PRISM version 6.0 (GraphPad, San Diego, CA). In the graphs, * \(P < 0.05\), ** \(P < 0.01\), *** \(P < 0.001\).
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Author contributions
I.B. designed, performed and interpreted the experiments and wrote the manuscript. F.L., F.M., V.V. and L.C. performed and assisted in the experiments. D.P. provided the prokr1b ct814/ct814 mutant line and interpreted the experiments. M.B., Y.G., and L.P. conceived the study, supervised the publication, interpreted the experiments and wrote the manuscript.

Competing interests
The authors declare no competing interests.

Additional information
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