Prokineticin receptor 2 affects GnRH3 neuron ontogeny but not fertility in zebrafish

Ivan Bassi1, Francesca Luzzani1, Federica Marelli1,2, Valeria Vezzoli1, Ludovica Cotellessa1,2, David A. Prober3, Luca Persani1,2, Yoav Gothilf4 & Marco Bonomi2✉

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), 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 model8,11. Among the genes involved in CHH, prokineticin receptor 2 (PROKR2) has an important role 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. Prokinetics 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 expenditure13–15. 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\(^{8,20,21}\), 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 \(tg(\text{gnrh3:EGFP})\) 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\(^{+/+}\) and WT siblings at these two time points (Fig. 4A, B, E, F), while prokr1b\(^{−/−}\) 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\(^{−/−}\) 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\(^{−/−}\), 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 lHβ and fSHβ in the brain (Fig. 6O) together with a strong decrease of the gonadotropin 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 ontogeny38-39. 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 close to the olfactory bulbs and appears similar to that of gnrh3 at 48 and 72 hpf. Accordingly, Real-Time PCR data show that during this time window prokr1b expression increases and then drastically decreases at 96 hpf. At 72 hpf, the development of GnRH3 fibers is complete and is followed by the migration of GnRH3 somata from the olfactory region to the hypothalamus. Consistent with the possibility that zebrafish prokr1b is an ortholog of human PROKR2, knockdown of prokr1b, but not prokr1a, affected the formation of rostral GnRH3 fibers

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.
fibers, similar to humans with PROKR2 mutations. Importantly, similar defects were also present in homozygous prokr1b mutant embryos at 48 hpf, and this phenotype was rescued by injection of WT prokr1b mRNA. Taken together, these results demonstrate that prokr1b is important for the correct migration of GnRH3 neuron fibers.

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 migration. Two recent studies conducted in zebrafish have highlighted differences in the role of the GnRH system during puberty and fertility. Li and colleagues showed that triple mutants lacking gnrh3 and the 2 kisspeptin ligands undergo normal puberty and gonadal maturation. 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 zebrafish. 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 zebrafish. 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 zebrafish. 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 modification. 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 activate, 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 suggested.

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 zebrafish. Indeed, despite the variable phenotypic features, the Tg(gnrh3:EGFP);prokr1b<sup>−/−</sup> line presented here suggests that prokr1b is the orthologue of human PROKR2, and demonstrates that its loss affects the development of GnRH1 neuronal fibers in zebrafish, asin humans, but also the expression of the lhr and fshr at the testes level, thus indicating a complex implication of the prokineticin 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 techniques. 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.
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. Wild-type (WT) zebrafish of the AB strain were obtained from the Wilson lab (University College London, London, United Kingdom). The tg (gnrh3:EGFP) and prokr1b<sup>ct814/ct814</sup> zebrafish lines have been previously described.

**Figure 6.** Histological sections conducted on 3 months of age fish showed no defects in the structure of gonads and testis between WT (A,C), heterozygous (E,G) and homozygous mutant (I,K) Sections zoomrevealed fully mature oocytes (black arrows in B,F,J) and fully mature spermatozoa (yellow arrows in D,H,L) in WT, prokr1b<sup>+</sup>/− and prokr1b<sup>−/−</sup> animals. Comparison of reproductive outputs (M) and GSI index (N) revealed no differences between WT, heterozygous and homozygous mutants. Real-Time PCR conducted on brain (O) and gonads (P) of WT and mutant fish showed a different expression of lhβ, fshβ, lhr and fshr in prokr1b<sup>−/−</sup> males. Image taken using Leica Application Suite software (LAS version 4.7.0; URL: https://www.leica-microsystems.com/products/microscope-software/p/leica-application-suite/).
Real-Time PCR. Reverse Transcription-Polymerase Chain Reaction (RT-PCR) was performed on total RNA prepared from 20 zebrafish oocytes and embryos for each developmental stage using the Total RNA Isolation Kit (Ambion, Thermo Fisher, Waltham MA) or the RNAgent 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. 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. PCR products were cloned into the pGEM-T Easy vector (Promega, Table S2). The cDNA-containing plasmids were linearized 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 MgSO4·H2O; 0.6 mM Ca(NO3)2·5 MHzepes pH 7.2) at 2 mM and stored at −80°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 H2O) 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/cct814 line and rescue experiments. We crossed tg(gnrh3:EGFP) animals to prokr1b<ct814/cct814 animals to generate the tg(gnrh3:EGFP); prokr1b<ct814/cct814 line. Fin clipping was performed to isolate genetic material from individual fish for genotyping accordingly to what previous published in Chen and colleague. Both were splice-blocking MOs synthesised by Gene Tools LLC (Oregon, USA) and targeted the human -globin β gene. Morpholinos were 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. S1). 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 H2O) 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.

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. 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 at 48 h or 72 hpf in UltraPure Low Melting Point Agarose (Thermo Fisher Scientific) and photographed using a confocal laser scanning microscope (Nikon C2) with a 20x objective.

Statistical analysis. Statistical analyses in Fig. 5 were 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.

Received: 19 October 2019; Accepted: 7 April 2020; Published: 06 May 2020

References
1. Calvin, J. L., Slater, C. H., Bolduc, T. G., Laudano, A. P. & Sower, S. A. Multiple molecular forms of gonadotropin-releasing hormone in the brain of an elasmobranch: evidence for IR-lamprey GnRH. Peptides 14, 725–729 (1993).
2. Uchida, K. et al. Evolutionary origin of a functional gonadotropin in the pituitary of the most primitive vertebrate, hagfish. Proc Natl Acad Sci USA 107, 15832–15837 (2010).
3. Takahashi, A., Kanda, S., Abe, T. & Oka, Y. Evolution of the hypothalamic-pituitary-gonadal axis regulation in vertebrates revealed by knockout medaka. Endocrinology 157, 3994–4002 (2016).
4. Lethimonier, C., Madigou, T., Munoz-Cueto, J. A., Lareyre, J. J. & Kah, O. Evolutionary aspects of GnRHs, GnRH neuronal systems and GnRH receptors in teleost fish. Gen Comp Endocrinol 135, 1–16 (2004).
5. Kavanagh, S. I., Nozaki, M. & Sower, S. A. Origins of gonadotropin-releasing hormone (GnRH) in vertebrates: identification of a novel GnRH in a basal vertebrate, the sea lamprey. Endocrinology 149, 3860–3869 (2008).
6. Foroni, P. E. & Wray, S. GnRH, anosmia and hypogonadotropic hypogonadism—where are we? Front Neuroendocrin. 36, 165–177 (2015).
7. Vezzoli, V. et al. The complex genetic basis of congenital hypogonadotropic hypogonadism. Minerva Endocrinol 41, 223–239 (2016).
8. Seminara, S. B., Hayes, F. J. & Crowley, W. F. Gonadotropin-Releasing Hormone Deficiency in the Human (Idiopathic Hypogonadotropic Hypogonadism Genetic Considerations. Endocr. Rev. 19, 521–539 (1998).
9. Boehm, U. Expert consensus document: European Consensus Statement on congenital hypogonadotropic hypogonadism—pathogenesis, diagnosis and treatment. Nat Rev Endocrinol 11, 547–564 (2015).
10. Bonomi, M. et al. Characteristics of a nationwide cohort of patients presenting with isolated hypogonadotropic hypogonadism (IHH). Eur J Endocrinol 178, 23–32 (2018).
11. Sykiotis, G. P. et al. Oligogenic basis of isolated gonadotropin-releasing hormone deficiency. Proc Natl Acad Sci USA 107, 15140–15144 (2010).
12. Soga, T. et al. Molecular cloning and characterization of prokinetin receptors. Biochem Biophys Acta 1579, 173–179 (2002).
13. Negri, L. et al. Bv8/Prokinetins and their Receptors A New Pronociceptive System. Int. Rev. Neurobiol. 85, 145–157 (2009).
14. Zhou, W., Li, J.D, Hu, W. P., Cheng, M. Y. & Zhou, Q. Y. Prokinetin 2 is involved in the thermoregulation and energy expenditure. Regul. Pept. https://doi.org/10.1016/j.regpep.2012.08.003 (2012).
15. Shojaei, F. et al. Bv8 regulates myeloid-cell-dependent tumour angiogenesis. Nature https://doi.org/10.1038/nature06348 (2007).
16. Matsunoto, S. et al. Abnormal development of the olfactory bulb and reproductive system in mice lacking prokinetin receptor PKR2. Proc Natl Acad Sci USA 103, 4140–4145 (2006).
17. Abreu, A. P., Kaiser, U. B. & Latronico, A. C. The role of prokinetins in the pathogenesis of hypogonadotropic hypogonadism. Neuroendocrinology 91, 283–290 (2010).
18. Libri, D. V. et al. Germline prokinetin receptor 2 (PROKR2) variants associated with central hypogonadism cause differential modulation of distinct intracerebral pathways. J Clin Endocrinol Metab 99, E458–63 (2014).
19. Dode, C. et al. Kisspeptin/kisspeptin receptors: a complex network for reproductive control. Front Neuroendocr. 34, 274–284 (2013).
20. Cole, L. W. et al. Mutations in prokinetin 2 and prokineticin receptor 2 genes in human gonadotrophin-releasing hormone deficiency: molecular genetics and clinical spectrum. J Clin Endocrinol Metab 93, 3551–3559 (2008).
21. Abreu, A. P. et al. Loss-of-function mutations in the genes encoding prokinetin-2 or prokineticin receptor-2 cause autosomal recessive Kallmann syndrome. J Clin Endocrinol Metab 93, 4113–4118 (2008).
22. Dode, C. & Rondard, P. PROK2/PROKR2 Signaling and Kallmann Syndrome. Front Endocrinol 4, 19 (2013).
23. Martin, C. et al. The role of the prokinetin receptor in human reproduction: evidence from the study of human and murine gene mutations. Endocr. Res. 32, 225–246 (2011).
24. Pitteloud, N. et al. Digenic mutations account for variable phenotypes in idiopathic hypogonadotropic hypogonadism. J Clin Invest 117, 457–463 (2007).
25. Lin, D. C. et al. Identification and molecular characterization of two closely related G protein-coupled receptors activated by prokineticins/endocrine gland vascular endothelial growth factor. J Biol Chem 277, 19276–19280 (2002).
26. Chen, S. et al. Light-Dependent Regulation of Sleep and Wake States by Prokineticin 2 in Zebrafish. J Biol Chem 283, 45980–45989 (2008).
27. Abraham, E. et al. Kallmann syndrome: mutations in the genes encoding prokinetin-2 and prokineticin receptor-2. PLoS Genet 2, e175 (2006).
28. Palevitch, O. et al. Nasal embryonic LHRH factor plays a role in the developmental migration and projection of gonadotropin-releasing hormone 3 neurons in zebrafish. Dev Dyn 238, 66–75 (2009).
29. Thiese, C., Thiase, B., Halpern, M. E. & Postlethwait, J. H. goosecoid Expression in neurectoderm and mesendoderm is disrupted in zebrafish cyclops gastrulas. Dev. Biol. 164, 420–429 (1994).
30. Schindelin, J. et al. Fiji: An open-source platform for biological-image analysis. Nature Methods https://doi.org/10.1038/nmeth.2019 (2012).
31. Gonzales, J. M. & Law, S. H. W. Feed and feeding regime affect growth rate and gonadosomatic index of adult zebrafish (Danio Rerio). Zebrafish https://doi.org/10.1080/15462225.2013.80891 (2013).
Acknowledgements
The study was supported by funds from IRCCS Istituto Auxologico Italiano (Ricerca Corrente funds: 05C623_2016) and funds from University of Milan – Dept. of Clinical Sciences and Community Health (Piano di sostegno alla ricerca - Linea 2 Azione B).

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 prokr1βct814/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
Supplementary information is available for this paper at https://doi.org/10.1038/s41598-020-64077-2.

Correspondence and requests for materials should be addressed to M.B.

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2020