Published in final edited form as:
J Neuroendocrinol. 2010 August ; 22(8): 944–950. doi:10.1111/j.1365-2826.2010.02024.x.

Compound Deficiencies in Multiple Fibroblast Growth Factor Signalling Components Differentially Impact the Murine Gonadotrophin-Releasing Hormone System

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Abstract

Gonadotrophin-releasing hormone (GnRH) neurones control the onset and maintenance of fertility. Aberrant development of the GnRH system underlies infertility in Kallmann syndrome [KS; idiopathic hypogonadotropic hypogonadism (IHH) and anosmia]. Some KS patients harbour mutations in the *fibroblast growth factor receptor 1 (Fgfr1)* and *Fgf8* genes. The biological significance of these two genes in GnRH neuronal development was corroborated by the observation that GnRH neurones were severely reduced in newborn transgenic mice deficient in either gene. In the present study, we hypothesised that the compound deficiency of *Fgf8* and its cognate receptors, *Fgfr1 and Fgfr3*, may lead to more deleterious effects on the GnRH system, thereby resulting in a more severe reproductive phenotype in patients harbouring these mutations. This hypothesis was tested by counting the number of GnRH neurones in adult transgenic mice with digenic heterozygous mutations in *Fgfr1/Fgf8*, *Fgfr3/Fgf8* or *Fgfr1/Fgfr3*. Monogenic heterozygous mutations in *Fgfr1, Fgf8* or *Fgfr3* caused a 30–50% decrease in the total number of GnRH neurones. Interestingly, mice with digenic mutations in *Fgfr1/Fgf8* showed a greater decrease in GnRH neurones compared to mice with a heterozygous defect in the *Fgfr1 or Fgf8* alone. This compounding effect was not detected in mice with digenic heterozygous mutations in *Fgfr3/Fgf8* or *Fgfr1/Fgfr3*. These results support the hypothesis that IHH/KS patients with digenic mutations in *Fgfr1/Fgf8* may have a further reduction in the GnRH neuronal population compared to patients harbouring monogenic haploid mutations in *Fgfr1* or *Fgf8*. Because only *Fgfr1/Fgf8* compound deficiency leads to greater GnRH system defect, this also suggests that these fibroblast growth factor signalling components interact in a highly specific fashion to support GnRH neuronal development.

Keywords

fibroblast growth factor receptor; fibroblast growth factor 8; gonadotrophin-releasing hormone neurones; hypogonadotropic hypogonadism; Kallmann syndrome

Centrally, reproductive fitness is determined by gonadotrophin-releasing hormone (GnRH) neurones, which are localised in the preoptic area and hypothalamus (1–3). Disruption of GnRH neuronal development results in severe clinical consequences that are primarily associated with reproductive failure. In humans, GnRH system deficiency is a hallmark of Kallmann syndrome (KS), which is a form of idiopathic hypogonadotropic hypogonadism

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The authors declare that there are no conflicts of interest.

(IHH) associated with anosmia. As expected, KS/IHH patients have nondetectable to low gonadotrophin and gonadal steroid hormone levels, ultimately resulting in the absence of pubertal onset and reproductive failure.

To date, KS has been causally linked to loss-of-function mutations in six genes: *anosmin-1*, *fibroblast growth factor receptor 1* (*Fgfr1*), *fibroblast growth factor 8* (*Fgf8*), *prokineticin receptor-2*, *prokineticin-2* or *Chd-7* (4–10). Of these gene mutations, the loss-of-function mutations in *Fgf* genes document the deleterious impact of fibroblast growth factor (FGF) signalling deficiency on the GnRH system and reproductive performance (11–13). Recently, we demonstrated that fibroblast growth factor receptor (FGFR) 1 is the FGF receptor especially critical for mouse GnRH neuronal development (11). *Fgfr1* hypomorphic mice, which express 30–60% lower levels of functional *Fgfr1* mRNA (14), showed approximately 88% reduction in the total number of GnRH neurones compared to wild-type littermates (11). By contrast, the GnRH neuronal system was unaffected in newborn *Fgfr3* knockout mice (11). Furthermore, studies showed that *Fgf8* hypomorphic mice, which exhibit a 65% reduction in *Fgf8* mRNA levels, suffered a loss in GnRH neurones similar to *Fgfr1* hypomorphs (11). The convergence of phenotype between *Fgf8* and *Fgfr1* hypomorphs suggests that FGFR1, not FGFR3, is the cognate receptor mediating the actions of FGF8 in the genesis of GnRH neurones (11).

A fraction of KS/IHH patients was shown to harbour heterozygous loss-of-function mutations in multiple genes (9, 15). For example, a proband who harboured digenic heterozygous mutations in *Fgfr1* and *Fgf8* was diagnosed with absent puberty (IHH). Of interest, the proband's father, who carried a monogenic heterozygous *Fgfr1* mutation, exhibited only delayed puberty (9). The most likely explanation for this observation is that the digenic presence of heterozygous mutations in *Fgfr1 and Fgf8* are additive during development, thereby impacting the GnRH neuronal system more deleteriously. To test this, we examined the GnRH system in transgenic mice harbouring several *Fgf* deficiencies. First, we examined whether the GnRH neuronal system is compromised in transgenic mice with monogenic heterozygous mutations in *Fgfr1*, *Fgfr3* or *Fgf8*. Second, we examined whether mice harbouring digenic heterozygous mutations in *Fgfr1/Fgf8*, *Fgfr3/Fgf8* or *Fgfr1/Fgfr3* exhibited compound deleterious effects on the GnRH neuronal system. The results obtained in the present study should allow us to more clearly define individual and additive roles of each of these three FGF signalling components in the development and maintenance of the GnRH system.

Materials and methods

Transgenic animals and crossbreeding

Fgfr1 hypomorphs (129sv/CD-1; obtained from Canadian Mutant Mouse Repository, Toronto, Ontario, Canada), *Fgfr3* knockout mice (Swiss-Webster/C57Bl/6; obtained from Jackson Laboratories, Bar Harbor, ME, USA) and *Fgf8* hypomorphs (129P2/OlaHsd*CD-1; obtained from Mouse Regional Resource Centers, Davis, CA, USA) (14, 16, 17) were housed in our animal facility under a 12 : 12 h light/dark cycle and fed ad *libitum*. Initially, transgenic mice were bred through heterozygote : heterozygote mating to obtain: wild-type and heterozygous ($^{+/-}$) *Fgfr1* hypomorphic mice, *Fgfr3* knockout mice or *Fgf8* hypomorphic mice. Subsequently, *Fgfr1* $^{+/-}$ were crossbred with *Fgf8* $^{+/-}$ mice to generate F_1 mice offspring harbouring digenic heterozygous mutations in *Fgfr1 and Fgf8* (*Fgfr1* $^{+/-}$ / *Fgf8* $^{+/-}$). Similarly, we crossbred *Fgfr3* $^{+/-}$ mice with *Fgf8* $^{+/-}$ mice, and *Fgfr1* $^{+/-}$ mice with *Fgfr3* $^{+/-}$ mice, to yield F_1 mice with digenic heterozygous mutations in *Fgfr3* and *Fgf8* (*Fgfr3* $^{+/-}$ / *Fgf8* $^{+/-}$)or *Fgfr1* and *Fgfr3* (*Fgfr1* $^{+/-}$ / *Fgfr3* $^{+/-}$). Within each crossbreeding, wild-type, *Fgfr1* $^{+/-}$, *Fgfr3* $^{+/-}$ or *Fgf8* $^{+/-}$ mice were also generated. All comparisons were made among siblings (wild-type, monogenic, digenic) with the same

hybrid genetic background to minimise the effect on the GnRH system as a result of strain differences. A polymerase chain reaction was performed on genomic DNA isolated from tail biopsies to identify genotype. All animal procedures complied with protocols as approved by the Institutional Animal Care and Use Committee at the University of Colorado.

GnRH immunocytochemistry

Adult mouse brains (2 months of age) were immersion-fixed in 4% paraformaldehyde/0.1 M phosphate buffer for 24 h at 4 °C. GnRH neurones were detected using the rabbit anti-GnRH polyclonal antibody LR5 (generously provide by Dr R. Benoit, Montreal General Hospital, Quebec, Canada) as previously described (11). Briefly, serial coronal sections of adult brains (40 μm) were incubated with LR-5 (1 : 10 000) diluted in 0.1 M phosphate buffered-saline/0.4% triton-X containing 4% normal donkey serum and 10% normal horse serum for 4 days at 4 °C. Subsequently, sections were incubated with a biotinylated-donkey anti-rabbit antibody (dilution 1 : 500; Jackson Laboratories, West Grove, PA, USA), avidin and biotin-coupled horseradish peroxidase (ABC kit; Vector Laboratories, Burlingame, CA, USA), and reacted with 0.05% 3,3′-diaminobenzidine/0.01% H_2O_2. Sections were mounted on gelatin-coated glass slides, dehydrated in increasing concentrations of ethanol, delipified with Histoclear (Fisher Scientific, Fair Lawn, NJ, USA), and covers-lipped with Permount (Fisher Scientific).

GnRH neuronal counts and statistical analysis

The number of GnRH-immunoreactive (IR) neurones was specifically quantified in serial coronal 40-μm sections from the anterior preoptic region to the posterior hypothalamus. Although this analysis did not include the rostral brain regions that are in the direct vicinity of the olfactory bulbs, the population of GnRH neurones residing within the regions quantified encompassed the vast majority of the mouse GnRH neuronal system. All slides were coded and scored by an individual who was blind to their identities. A GnRH-IR neurone was scored positive only if it also showed a clear cell nucleus. GnRH neuronal counts from both males and females were pooled in our analysis because the number of GnRH-IR neurones did not differ between the sexes in our hands. To analyse the distribution of GnRH neurones, the beginning of the organum vasculosum lamina terminalis (OVLT) was designated as section 0. Subsequently, 20 sections prior to and after section 0 were included in the analysis. One-way ANOVA followed by the Student–Newman–Keuls test or Student's t-test was used to test for significant differences. $P < 0.05$ was considered statistically significant.

Results

GnRH system deficit in *Fgfr1* $^{+/-}$, *Fgfr3* $^{+/-}$ and *Fgf8* $^{+/-}$ mice

Student t-tests indicated that adult mice harbouring monogenic heterozygous mutations in *Fgfr1*, *Fgfr3* or *Fgf8* had significantly reduced GnRH-IR neurones in the preoptic/hypothalamus region compared to wild-type. The total number GnRH-IR neurones in *Fgfr1* $^{+/-}$ mice (372 ± 48.4; n = 7) mice was significantly lower than in wild-type *Fgfr1* mice (539 ± 30.1; n = 8; P = 0.046; Fig. 1). Similarly, *Fgf8* $^{+/-}$ mice (214 ± 18.3; n = 4) have significantly less GnRH-IR neurones than wild-type *Fgf8* mice (424 ± 29.1; n = 4; P < 0.01; Fig. 1B), which replicated our earlier findings (11). The number of GnRH-IR neurones was also significantly lower in *Fgfr3* $^{+/-}$ mice (245 ± 36.8; n = 5) than in wild-type *Fgfr3* mice (426 ± 24.9; n = 6; P = 0.01; Fig. 1C). No gross morphological aberrations in the brain of these transgenic animals were detected (data not shown). Moreover, no marked reduction in fertility was detected in adult *Fgfr1* $^{+/-}$, *Fgfr3* $^{+/-}$ or *Fgf8* $^{+/-}$ mice at 2 months of age (data not shown).

Exacerbation of GnRH system deficit in Fgfr1 $^{+/-}$ / *Fgf8* $^{+/-}$ mice

One-way ANOVA showed that the total number of GnRH-IR neurones in the preoptic/hypothalamic region of mice with monogenic or digenic heterozygous mutations in *Fgfr1* and/or *Fgf8* was significantly different (i.e. higher or lower) among genotypes (i.e. wild-type, *Fgfr1* $^{+/-}$, *Fgf8* $^{+/-}$ and *Fgfr1* $^{+/-}$ / *Fgf8* $^{+/-}$; $P < 0.0001$; Fig. 2A–E). Post-hoc analysis showed that the number of GnRH-IR neurones was significantly lower in *Fgfr1* $^{+/-}$ (407 ± 40.0; n = 5), *Fgf8* $^{+/-}$ (347 ± 32.5; n = 7) or *Fgfr1* $^{+/-}$ / *Fgf8* $^{+/-}$ (215 ± 39.0; n = 5) mice compared to wild-type (601 ± 41.5; n = 7; Fig. 2E). *Fgfr1*$^{+/-}$ / *Fgf8* $^{+/-}$ mice had more GnRH neuronal loss compared to *Fgfr1* $^{+/-}$ or *Fgf8* $^{+/-}$ mice (Fig. 2E). There was no significant difference between *Fgfr1* $^{+/-}$ and *Fgf8* $^{+/-}$ mice. No gross morphological aberrations in the brain of these transgenic animals were detected (data not shown). The distribution of GnRH neurones in the fore-brain in mice of all genotypes retained the basic pattern of having more GnRH neurones near the OVLT (Fig. 2F).

GnRH system deficit was not exacerbated in *Fgfr3*$^{+/-}$ / *Fgf8*$^{+/-}$ mice

One-way ANOVA showed that the total number of GnRH-IR neurones in the preoptic/hypothalamic region of mice with monogenic or digenic heterozygous mutations in *Fgfr3* and/or *Fgf8* was different among genotypes ($P < 0.0001$; Fig. 3A). Post-hoc analysis showed that the total number of GnRH-IR neurones in the preoptic/hypothalamic region was significantly lower in *Fgfr3* $^{+/-}$ (270 ± 8.1; n = 6), *Fgf8* $^{+/-}$ (234 ± 9.4; n = 5) and *Fgfr3* $^{+/-}$ / *Fgf8* $^{+/-}$ (227 16.3; n = 7) mice compared to wild-type (443 ± 21.9; n = 7; Fig. 3A). There were no differences among *Fgfr3* $^{+/-}$, *Fgf*8$^{+/-}$ and *Fgfr3* $^{+/-}$ / *Fgf8* $^{+/-}$ mice. No gross morphological aberrations in the brain of these transgenic animals were detected (data not shown).

GnRH system deficit was not exacerbated in *Fgfr1* $^{+/-}$ / *Fgfr3* $^{+/-}$ mice

One-way ANOVA showed that the total number of GnRH-IR neurones in the preoptic/hypothalamic regions of mice with monogenic or digenic heterozygous mutations in *Fgfr1* and/or *Fgfr3* was different among genotypes ($P < 0.05$; Fig. 3B). Post-hoc analysis showed that the total number of GnRH-IR neurones in the preoptic/hypothalamic region was significantly lower in *Fgfr1* $^{+/-}$ (367 ± 27.7; n = 6), *Fgfr3* $^{+/-}$ (442 ± 47.9; n = 5) and *Fgfr1* $^{+/-}$ / *Fgfr3* $^{+/-}$ (394 ± 33.5; n = 5) mice compared to wild-type (566 ± 47.5; n = 5; Fig. 3B). There were no differences among *Fgfr1* $^{+/-}$, *Fgfr3* $^{+/-}$ and *Fgfr1* $^{+/-}$ / *Fgfr3* $^{+/-}$ mice. No gross morphological aberrations in the brain of these transgenic animals were detected (data not shown).

Discussion

Two novel findings emerged from the present study. First, monogenic *Fgfr3* haploinsufficiency reduced approximately one-half of the GnRH neuronal population in adult mice. Second, contrary to what one might be predicted, not all compound deficiencies in FGF signalling lead to more deleterious effects on the GnRH system than monogenic deficiencies alone. A greater GnRH system defect was seen only when *Fgfr1* and *Fgf8* deficiencies were combined. The latter finding supports the hypothesis that the presence of digenic mutation in *Fgfr1* and *Fgf8* may actually result in a further loss of GnRH neurones, ultimately contributing to the reported increase in the severity of reproductive symptoms (9, 15).

In the present study, we found that the number of GnRH neurones was higher in wild-type mice when they contained the 129sv/CD-1 background (*Fgfr1* hypomorphic mice). This discrepancy is likely a result of the inherently higher number of GnRH neurones in this hybrid background. To minimise the effect on the GnRH system as a result of strain

differences, we limited our comparisons between siblings (wild-type, monogenic, digenic) who shared similar hybrid genetic background. We acknowledge that even littermates may have variable genetic background in this hybrid breeding scheme but, given that the phenotypes associated with FGF signalling deficiency are highly penetrant (14, 16, 17), and that our data are consistent and statistically significant, it is unlikely that reduced GnRH neurones in FGF signalling-deficient mice resulted by chance simply from differences in their genetic background.

We previously showed that the GnRH system was unaffected in newborn homozygous *Fgfr3* knockout mice (*Fgfr3*$^{-/-}$) (11); thus, the current observation that adult *Fgfr3* $^{+/-}$ mice have a significant reduction in the number of GnRH-IR neurones is unexpected. At present, complete data on the GnRH system of adult *Fgfr3*$^{-/-}$ and newborn *Fgfr3* $^{+/-}$ mice are unavailable; thus, it is difficult to fully explain the role of *Fgfr3*. However, two possibilities exist. First, there may be an age-dependent decline of GnRH neurones in mice harbouring *Fgfr3* deficiency. This decline is only manifested shortly after birth, and thus the diminished GnRH neuronal population was only seen in adults (i.e. present study) but not in newborn mice (9) deficient in Fgfr3 signalling. In this regard, Fgfr3 could be more involved in the postnatal survival and/or differentiation of GnRH neurones than their development. Supporting this hypothesis, transgenic mice with reduced FGF responsiveness in GnRH neurones (13) have been shown to undergo a postnatal age-dependent decline in the number of GnRH neurones (18). Second, when *Fgfr3* is genetically ablated, as in the case of *Fgfr3*$^{-/-}$ mice, the expression of other *Fgfrs* such as *Fgfr1* may developmentally up-regulate to compensate. This compensation could lead to an absence of GnRH system defect in *Fgfr3*$^{-/-}$ mice as observed previously (11). Regardless, the data obtained in the present study are the first to show that *Fgfr3* haploinsufficiency significantly compromises the adult GnRH system. *Fgfr3* thus becomes the third FGF signalling gene that, when mutant, leads to an aberrant GnRH system in the mouse (11, 13). These data provide a strong impetus for exploring the role of *Fgfr3* as a candidate gene for IHH/KS.

A study in one X-linked KS foetus showed that GnRH neurones do not reach the preoptic/hypothalamus regions as a result of migratory failure (19). However, previous data indicated migratory failure is unlikely the cause for GnRH system defects in mice with *Fgf8* and *Fgfr1* deficiencies. Indeed, extensive analysis of the newborn nasal and brain region of *Fgfr1*$^{-/-}$ and *Fgf8*$^{-/-}$ hypomorphs did not detect aberrantly localised GnRH neurones (11). Furthermore, transgenic mice with reduced FGF responsiveness in GnRH neurones exhibited normal GnRH neuronal migration into the fore-brain (13). Instead, we showed that GnRH neurones simply did not emerge from the olfactory placode of *Fgf8*$^{-/-}$ hypomorphic embryos, suggesting Fgf8-dependent activation of Fgfr1 was required for the genesis of GnRH neurones (11). Together, these results strongly suggest that the reduced GnRH-IR neurones in *Fgfr1* $^{+/-}$, *Fgf8* $^{+/-}$ or *Fgfr1* $^{+/-}$ / *Fgf8* $^{+/-}$ mice result from the developmental elimination of GnRH neurones rather than migratory failure.

In the present study, we found that approximately 60–70% of GnRH neurones are missing in the preoptic area/hypothalamus of *Fgfr1*- and/or *Fgf8*-deficient mice. It is unknown how this GnRH neuronal loss impacts the function of the postnatal mouse GnRH system, and whether GnRH neuronal loss in specific brain regions differentially affects reproductive function. Previous studies in rodents have shown that GnRH neurones at the level of the OVLT are especially important for the preovulatory luteinising hormone/follicle-stimualting hormone (LH/FSH) surge (20–22). Indeed, destruction of the OVLT, which results in a striking reduction in GnRH content in the OVLT and median eminence, also causes a dramatic attenuation of the preovulatory LH/FSH surge (23). In our laboratory, adult *Fgfr1* $^{+/-}$ or *Fgf8* $^{+/-}$ mice do not exhibit a marked reduction in fertility, although this does not necessarily indicate the preovulatory LH/FSH surge is not compromised. Currently, we are

investigating whether the preovulatory LH/FSH surge in *Fgfr1* $^{+/-}$, *Fgf8* $^{+/-}$, and *Fgfr1* $^{+/-}$ / *Fgf8* $^{+/-}$ female mice exhibit marked deficits.

On the basis of our data, we have constructed a working model that may explain why only the *Fgfr1*/*Fgf8* combination was effective in exacerbating GnRH system defects (Fig. 4). First, we hypothesise that the simultaneous presence of heterozygous deficiencies in *Fgfr1* and *Fgf8* impact the same GnRH precursor population at the same time to enhance the severity of GnRH neuronal fate specification defects, leading to additive effects when both are mutant. This hypothesis is well supported by our previous findings that Fgf8 acts through Fgfr1 to stimulate the genesis of GnRH neurones (11). Second, we hypothesise that *Fgf8* and *Fgfr3* deficiencies are not additive because they affect the same GnRH neuronal population at different times. In other words, *Fgf8* deficiency obliterates a population of GnRH precursor cells that subsequently becomes Fgfr3-dependent after fate specification. Because this population is already lost when *Fgf8* is deficient, an additional reduction in Fgfr3 signalling has no further effect. The second hypothesis is based strictly on our present observations and requires future validation. However, the second hypothesis is consistent with our present data suggesting a later involvement of *Fgfr3* in the postnatal maintenance of the GnRH system. Similarly, *Fgfr1* and *Fgfr3* deficiencies are not additive because they may impact the same GnRH neuronal population at different times, with the former affecting GnRH precursors before fate specification and the latter after fate specification. This assumption again requires further validation. Overall, our model proposes that different combinations of *Fgfr*/*Fgf* exert different effects on the GnRH neuronal system in a sequential and temporally specific fashion. This specific nature of FGF action was supported by the findings that FGF ligands and receptors are sequentially expressed in the olfactory region during a developmental time frame consistent with the emergence and migration of GnRH neurones (24, 25).

The olfactory placode, from where mouse GnRH neurones are derived, expresses Fgfr1, FGFR2 and Fgfr3 proteins (26). Nonetheless, previous studies have shown that fate specification of GnRH precursor cells requires Fgf8 signalling that is mediated by Fgfr1 instead of Fgfr3 (11). Fgf8-dependent Fgfr1 activation may enhance the survival rate of GnRH precursor cells. Indeed, previous studies have shown that FGFRs induce a myriad of intracellular signal transduction pathways including the PI3K/AKT pathway, which protects against apoptotic cell death (27, 28). Therefore, the loss of GnRH neurones in mice harbouring monogenic heterozygous *Fgfr1*/*Fgf8* deficiencies may be the result of a decrease in cell survival, and this defect may be further enhanced in mice with digenic heterozygous *Fgfr1*/*Fgf8* deficiencies. A similar mechanism may also cause the loss of GnRH neurones found in *Fgfr3* deficient mice, albeit after GnRH neurones have become fate specified.

In conclusion, the present study has produced two major findings. First, Fgfr3 is important for the normal development and/or maintenance of the mouse GnRH neuronal population, possibly by exerting its effects after GnRH neuronal fate specification. Consequently, *Fgfr3* is a candidate gene that may be involved in the pathogenesis of IHH/KS. Second, not all compound deficiencies in FGF signalling lead to the exacerbation of the deleterious effects on the GnRH system compared to monogenic deficiencies alone. Only the combination of *Fgfr1* and *Fgf8* deficiencies caused greater deleterious effects on the GnRH neuronal population. The latter finding illustrates the highly complex and specific nature in which FGF signalling components interact to promote the emergence and maintenance of the GnRH system.

Acknowledgments

This study was supported by NIH RO1 HD042634, the Endocrine Society Bridge Award to P.S.T and NIH K99HD058044 to W.C.J.C.

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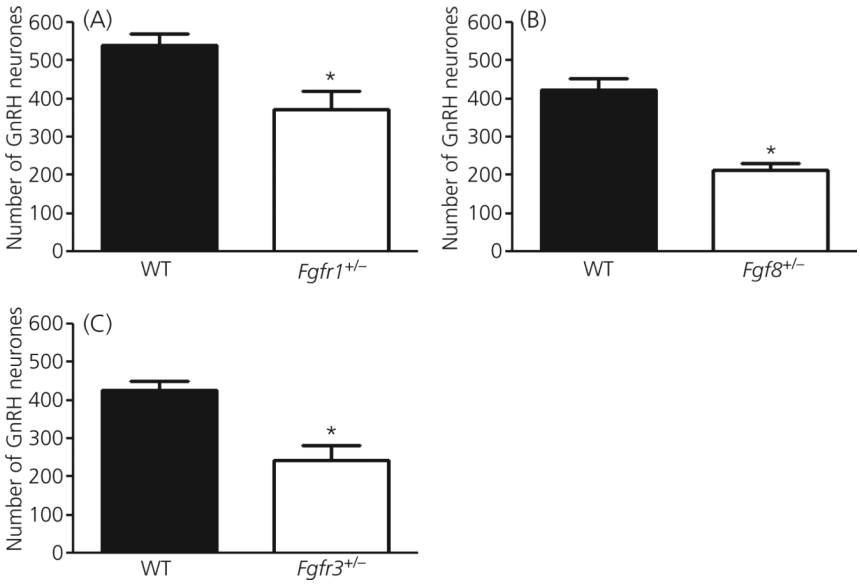

Fig. 1.

The number of gonadotrophin-releasing hormone (GnRH) neurones in the preoptic/hypothalamus region of mice with monogenic heterozygous defects in fibroblast growth factor signalling components. (A) *Fgfr1* $^{+/-}$ mice (n = 7) have significantly fewer GnRH neurones than wild-type (WT) mice (n = 8; P = 0.046). (B) *Fgf8* $^{+/-}$ mice (n = 4) have significantly fewer GnRH neurones than wild-type mice (n = 4; P < 0.02). (C) *Fgfr3* $^{+/-}$ mice (n = 5) have significantly fewer GnRH neurones than wild-type mice (n = 6; P = 0.01). Asterisks represent significant differences. Each bar represents the mean ± SEM.

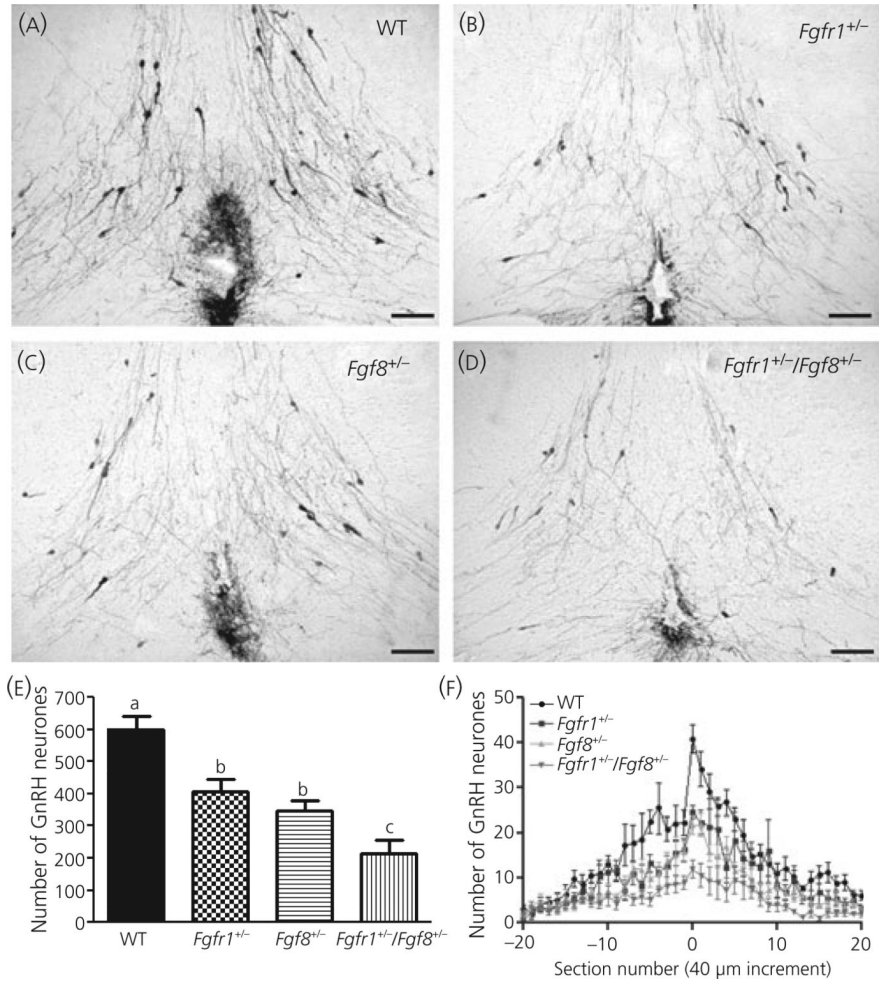

Fig. 2.

Gonadotrophin-releasing hormone (GnRH) system in *Fgfr1* $^{+/-}$, *Fgf8* $^{+/-}$ or *Fgfr1* $^{+/-}$ / *Fgf8* $^{+/-}$ mice. Representative photomicrographs showing GnRH-immunoreactive (IR) neurones at the level of the organum vasculosum lamina terminalis (OVLT) of (A) wild-type (WT) (n = 7), (B) *Fgfr1* $^{+/-}$ (n = 5), (C) *Fgf8* $^{+/-}$ (n = 7) and (D) *Fgfr1* $^{+/-}$ / *Fgf8* $^{+/-}$ (n = 5) mice. Note that the number of GnRH-IR neurones is reduced in *Fgfr1* $^{+/-}$, *Fgf8* $^{+/-}$ and *Fgfr1* $^{+/-}$ / *Fgf8* $^{+/-}$ mice compared to wild-type mice. Moreover, the reduction in the number of GnRH-IR neurones in *Fgfr1* $^{+/-}$ / *Fgf8* $^{+/}$ mice is visually more prominent compared to *Fgfr1* $^{+/-}$ or *Fgf8* $^{+/-}$ mice. Scale bar = 100 μm. (E, F) Total number and distribution of GnRH neurones in mice deficient in *Fgfr1* and/or *Fgf8*.(E) ANOVA followed by post-hoc test showed the total number of GnRH-IR neurones in the preoptic/hypothalamus was significantly lower in *Fgfr1* $^{+/-}$ (n = 5), *Fgf8* $^{+/-}$ (n = 7) or *Fgfr1* $^{+/-}$ / *Fgf8* $^{+/-}$ (n = 5) mice than in wild-type (n = 7) mice ($P < 0.0001$ by ANOVA). Moreover, the loss of GnRH neurones was greater in *Fgfr1* $^{+/-}$ / *Fgf8* $^{+/-}$ mice than in *Fgfr1* $^{+/-}$ and *Fgf8* $^{+/-}$ mice. (F) The rostral–caudal distribution of GnRH neurones in wild-type, *Fgfr1* $^{+/-}$, *Fgf8* $^{+/-}$ and *Fgfr1* $^{+/-}$ / *Fgf8* $^{+/-}$ mice showed that mice of all genotypes retained the basic pattern of having more GnRH neurones near the OVLT. Each data point represents the mean ± SEM.

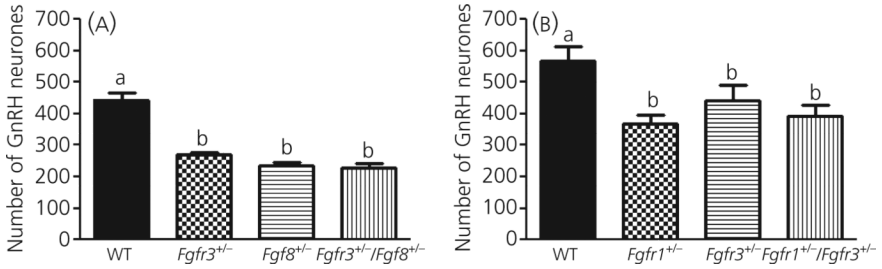

Fig. 3.

The number of gonadotrophin-releasing hormone (GnRH) neurones in the preoptic/hypothalamus region of mice with digenic or monogenic heterozygous deficiencies in various fibroblast growth factor signalling components. (A) ANOVA followed by post-hoc test showed GnRH neuronal number was significantly lower in *Fgfr3* $^{+/-}$ (n = 6), *Fgf8* $^{+/-}$ (n = 5) or *Fgfr3* $^{+/-}$ / *Fgf8* $^{+/-}$ (n = 7) mice compared to wild-type mice (WT) (n = 7; $P < 0.0001$ by ANOVA). (B) GnRH neuronal number was significantly lower in *Fgfr1* $^{+/-}$ (n = 6), *Fgfr3* $^{+/-}$ (n = 5) or *Fgfr1* $^{+/-}$ / *Fgfr3* $^{+/-}$ (n = 5) mice compared to wild-type mice (n = 5; $P < 0.0001$ by ANOVA). Different letters represent significant differences. Each bar represents the mean ± SEM.

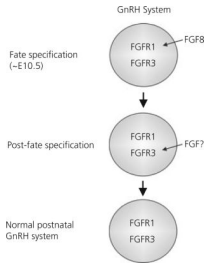

Fig. 4.

Hypothetical model illustrating the temporal sequence in which fibroblast growth factor (FGF) signalling components may affect gonadotrophin-releasing hormone (GnRH) neuronal development. In this model, we hypothesise that Fgf8-dependent activation of fibroblast growth factor receptor (FGFR) 1 is required for GnRH neuronal fate specification [i.e. approximately embryonic day (E) 10.5]. After fate specification, the same population of GnRH neurones becomes dependent on Fgfr3 for maintenance (11). Thus, deficiencies in *Fgfr1*, *Fgf8* or *Fgfr3* alone could compromise the GnRH system by affecting genesis (*Fgfr1* and *Fgf8*) or maintenance (*Fgfr3*). *Fgfr3* mutation in addition to *Fgfr1* or *Fgf8* did not further worsen the GnRH system defect because the target population was already absent as a result of earlier fate specification failure. Conversely, because Fgfr1 and Fgf8 both act on the fate specification stage, their effects can be additive. At present, it is unclear which FGF ligand(s) is required for the activation of Fgfr3 at the post-fate specification stage. Cumulatively, the data obtained in the present study suggest that the proper development of the mouse GnRH system requires the intricate orchestration of multiple FGF/FGFR signals in a time-specific manner.