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Fgfr2 and Fgfr3 are not required for patterning and maintenance of the midbrain and anterior hindbrain

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Abstract

The mid-/hindbrain organizer (MHO) is characterized by the expression of a network of genes, which controls the patterning and development of the prospective midbrain and anterior hindbrain. One key molecule acting at the MHO is the fibroblast growth factor (Fgf) 8. Ectopic expression of Fgf8 induces genes that are normally expressed at the mid-/hindbrain boundary followed by the induction of midbrain and anterior hindbrain structures. Inactivation of the *Fgf receptor* (*Fgfr*) *1* gene, which was thought to be the primary transducer of the Fgf8 signal at the MHO, in the mid-/hindbrain region, leads to a deletion of dorsal structures of the mid-/hindbrain region, whereas ventral tissues are less severely affected. This suggests that other Fgfrs might be responsible for ventral mid-/hindbrain region development. Here we report the analysis of *Fgfr2* conditional knockout mice, lacking the *Fgfr2* in the mid-/hindbrain region and of *Fgfr3* knockout mice with respect to the mid-/hindbrain region. In both homozygous mouse mutants, patterning of the mid-/hindbrain region is not altered, neuronal populations develop normal and are maintained into adulthood. This analysis shows that the Fgfr2 and the Fgfr3 on their own are dispensable for the development of the mid-/hindbrain region. We suggest functional redundancy of Fgf receptors in the mid-/hindbrain region.

Keywords: Fibroblast growth factor receptor; Midbrain; Hindbrain; Fgf signaling; Functional redundancy

Introduction

Regionalization of the mouse brain during embryogenesis strongly depends on organizing centers in the developing neural tube. One of the best-characterized organizing centers is the mid-/hindbrain organizer (MHO), located at the mid-/hindbrain boundary. The MHO is responsible for patterning of the future midbrain and anterior hindbrain (rhombomere 1) (Joyner, 1996; Wassef and Joyner, 1997; Liu and Joyner, 2001a, 2001b; Echevarria et al., 2003; reviewed in Wurst and Bally-Cuif, 2001; Rhinn and Brand, 2001; Prakash and Wurst, 2004; Nakamura and Watanabe, 2005). Its inductive and maintenance properties have been demonstrated by transplantation experiments. When tissue originating from the mid-/hindbrain boundary is placed into the diencephalon or caudal hindbrain ectopic mid- and anterior hindbrain structures are induced (Martinez et al., 1991, 1995). Further studies have shown that the MHO is characterized by the spatio-temporally tightly controlled expression of certain transcription factors, e.g. *Otx2*, *Gbx2*, *Pax2*, *Pax5*, *En1*, *En2* and the secreted molecules *Fgf8*, *Fgf15*, *Fgf17*, *Fgf18* and *Wnt1* (reviewed in Wurst and Bally-Cuif, 2001; Raible and Brand, 2004; Prakash and Wurst, 2004).

In order to determine whether one of these factors is instrumental in mediating the MHO activity, gene inactivation

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experiments for *Pax2/5*, *En1/2*, *Wnt1* and *Fgf8* have been performed and resulted in an early loss of the entire mid-/hindbrain region, which was preceded by the loss of MHO gene expression (McMahon and Bradley, 1990; Thomas and Capecchi, 1990; Wurst et al., 1994; Millen et al., 1994; Chi et al., 2003; Liu and Joyner, 2001b; Ye et al., 2001; Bouchard et al., 2000; Schwarz et al., 1997; reviewed in Prakash and Wurst, 2004). Subsequent gain-of-function (GOF) experiments, however, revealed that only Fgf8 is able to mimic the properties of the MHO. FGF8-coated beads are able to ectopically induce the expression of *Gbx2*, *En1/2*, *Pax2/5* and *Wnt1* and also the formation of midbrain and r1-structures (Crossley et al., 1996; Martinez et al., 1999; Liu et al., 1999; Liu and Joyner, 2001b). The patterning activity is intrinsic to the "b" isoform of Fgf8, whereas Fgf8a is responsible for the proliferation of the midbrain (Lee et al., 1997; Liu et al., 1999, 2003; Sato et al., 2001).

Although it is known that Fgf signals are transmitted by Fgf receptors (Fgfrs), the knowledge about the Fgfrs transmitting the Fgf8b signal at the mid-/hindbrain boundary is very limited. There is experimental evidence that the Fgfr1 mediates the majority of the Fgf8 signal in mid-/hindbrain development. The creation of a conditional *Fgfr1* mutant mouse, in which *Fgfr1* was specifically inactivated in the mid-/hindbrain region using an $En1^{Cre/+}$ mouse (Kimmel et al., 2000), led to a deletion of the inferior colliculi and the cerebellar vermis in the dorsal mid-/hindbrain region. However, ventral parts remained largely unaffected (Trokovic et al., 2003; Jukkola et al., 2006). This is in clear contrast to the phenotype of one of the conditional *Fgf8* knockout (KO) mouse models (Chi et al., 2003), in which *Fgf8* was inactivated in the mid-/hindbrain region using the same $En1^{Cre/+}$ mouse line. In this conditional *Fgf8* KO mouse, the complete mid-/hindbrain region is deleted. One likely explanation for these two contrasting phenotypes is the activity of other Fgfrs in the mid-/hindbrain region. In fact, Fgf8b can bind to the "c" splice isoforms of Fgfr1, Fgfr2 and Fgfr3 as well as to Fgfr4 although the affinity to each of these receptors is still under discussion (MacArthur et al., 1995; Olsen et al., 2006). Interestingly and in contrast to former studies, we could recently show that *Fgfr2* and *Fgfr3* are both expressed in the ventral mid-/hindbrain region of the developing mouse embryo (Blak et al., 2005). Therefore, in the conditional *Fgfr1* knockout mouse, the mild ventral phenotype may be due to the Fgfr2 and/or Fgfr3 as the receptors mediating Fgf8b activity in this region or at least acting in a redundant manner to the Fgfr1.

In order to elucidate the role of these Fgfrs in the mid-/hindbrain region, we investigated two different mouse models: a conditional KO of the *Fgfr2* ($En1^{Cre/+}$ $Fgfr2^{lox/lox}$) encompassing a deletion of *Fgfr2* in the midbrain and anterior hindbrain and the $Fgfr3^{-/-}$ mouse, carrying a null allele of *Fgfr3* (Colvin et al., 1996).

Both mouse mutants survived into adulthood. Interestingly, the analysis of both mutant mice at embryonic stages as well as in adulthood showed no alterations in patterning, histological integrity and identity of neuronal populations of the mid-/hindbrain region. Since Fgf signaling in these mice is also unaffected and expression of the other Fgf receptors is unchanged, we suggest that the Fgfr1/Fgfr3 and Fgfr1/Fgfr2 do rescue the loss of function of Fgfr2 and Fgfr3, respectively.

Materials and methods

Generation and genotyping of mutant mice

$Fgfr2^{lox/lox}$ mice were bred on an SV129 background. To generate the conditional knockout mice $En1^{Cre/+}$ $Fgfr2^{lox/lox}$, male $En1^{Cre/+}$ $Fgfr2^{lox/+}$ mice (on a C57/Bl6 background) were crossed with $Fgfr2^{lox/lox}$ females. Animals for analysis were obtained by brother–sister breeding in a C57/Bl6//SV129 background.

The $Fgfr2^{lox}$ allele was detected with primers located on both sides of the loxP sites flanking exon 5: sense primer: CTAGGCCAGCTGGACCAGAC; antisense primer: CGTTCTCTGATGGGCCATTG (location in DNA sequence see Fig. 1A).

Generation and genotyping of the $Fgfr3^{-/-}$ and $En1^{Cre/+}$ mice has been described previously (Colvin et al., 1996; Kimmel et al., 2000; Puelles et al., 2004).

Mice were maintained at the GSF mice core facility, Germany and the Institute of Biotechnology, University of Helsinki, Finland. The animal experiments were conducted under federal guidelines for the use and care of laboratory animals and were approved by the GSF Institutional Animal Care and Use Committee and by the committee of experimental animal research of the University of Helsinki.

mRNA in situ hybridization (ISH) analyses

Noon of the day of vaginal plug detection was designated E0.5. Timed pregnant female mice were killed by cervical dislocation. Embryos were dissected in PBS and immersion-fixed in 4% PFA over night. Adult mice (average age 4 months) were perfused transcardially with 4% PFA and brains were post-fixed in 4% PFA over night. Embryos and adult brains were paraffin embedded and sectioned on a microtome (Microm, Walldorf, Germany) in 8-μm-thick sections.

Radioactive *in situ* hybridizations were performed on sections of mouse embryos according to a modified version of the procedure described by Dagerlind et al. (1992). Antisense mRNA probes were transcribed from plasmids containing fragments of the murine tyrosine hydroxylase (*TH*) gene (GenBank accession number M69200), the serotonin-transporter gene (*Sert*) (GenBank accession number AF013604), *Otx2*, *Fgf8* (Broccoli et al., 1999), *Gbx2*, *Islet1*, *Pou4f1* (Puelles et al., 2003; GenBank accession number NM_010262, NM_021459, NM_011143), *Erm* (IMAGE-clone 3674281) and *GAD65* (GenBank accession number BC018380). The *Fgfr2* exon 5 was used as a probe after cloning of the PCR-amplified exon 5 into the TOPO 2 vector (Invitrogen) (GenBank accession number NCBI Y16155) using the genotyping primers.

Furthermore, the *En1* probe was a gift from A. Joyner (GenBank accession number NM_010133), the *Shh* probe was a gift from A. McMahon (GenBank accession number BC063087), the *Wnt1* probe was a gift from M. Wassef (GenBank accession number NM_021279), the *Pax2* probe was a gift from H. Fickenscher (GenBank accession number NM_011037), the *Spry1* probe was a gift from G. Martin (GenBank accession number NM_011896), the *Sef1* probe was a gift from R. Friesel (GenBank accession number AF459444), the *Mkp3* probe was a gift from J.A.Belo (GenBank accession number BC003869), the *Fgfr1* probe was a gift from R. Lauster (GenBank accession number NM_010206), the *Fgfr2* probe (TK domain) was a gift from C. Dickson (BC091652), the *Fgf17*, *Fgf18* and *Fgfr3* probes were gifts from D. Ornitz (GenBank accession number NM_008004, NM_008005, NM_008010), the *Nkx2.2* probe was a gift from D. Hartigan (GenBank accession number NM_010919) and the *Nkx6.1* probe was a gift from E. Puelles (GenBank accession number NM_144955/AF291666).

Non-radioactive *in situ* hybridizations were performed on 8-μm-thick horizontal paraffin section of adult mouse brains using a modified protocol from

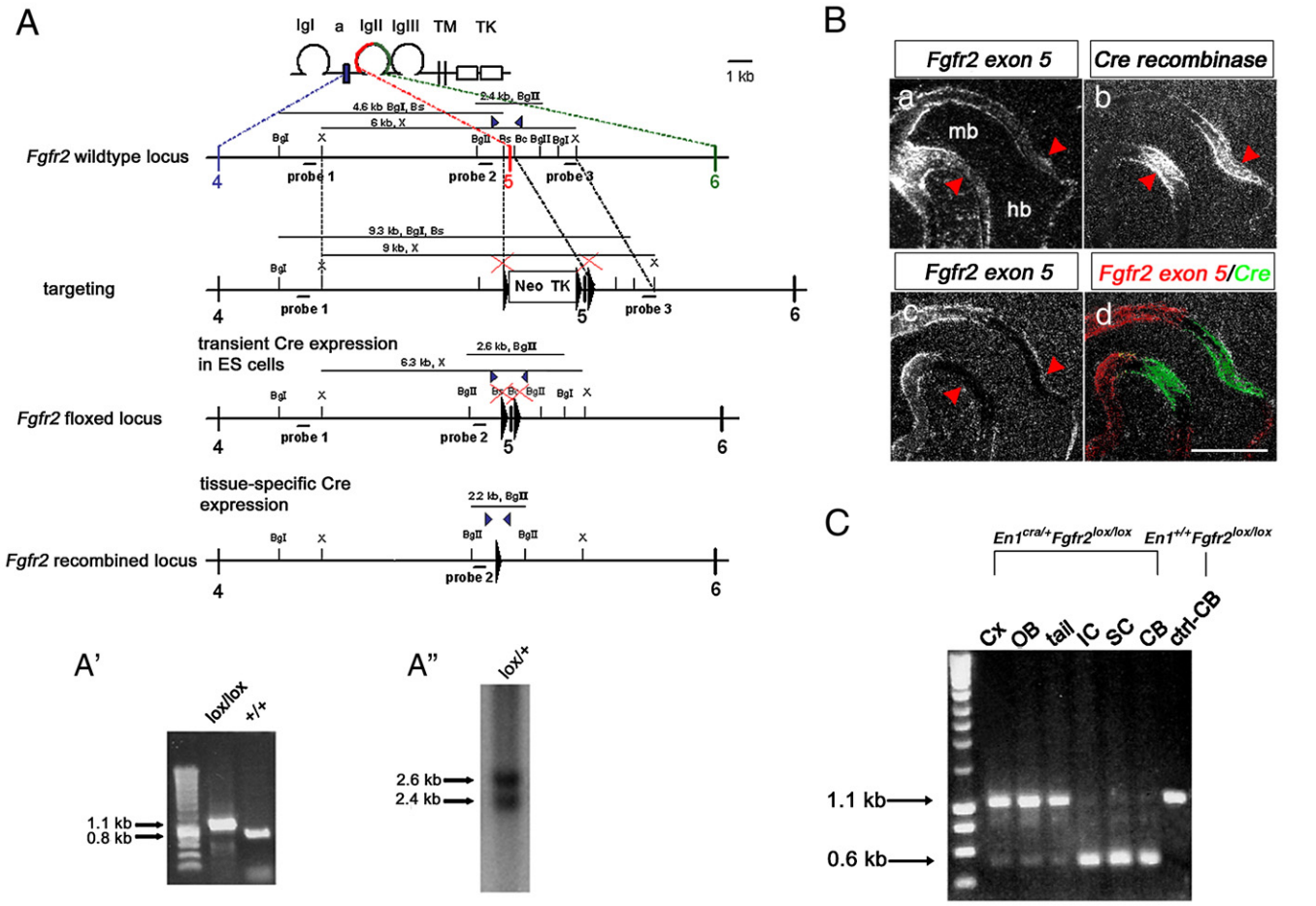

Fig. 1. (A) Generation of the conditional *Fgfr2* allele, $Fgfr2^{lox}$. The *Fgfr2* wild-type locus (exons 4, 5 and 6) is shown at the top. Exon 4 encodes the acidic box (a) of the Fgfr2 protein (blue), exon 5 encodes the first part of Ig-like domain II (red) and exon 6 encodes the second part (green). The scale bar of 1 kbp corresponds to the wild-type allele. The construct that was used for homologous recombination in ES cells was generated by introduction of a Neo TK cassette-itself flanked by loxP sites, 3′ of exon 5 and an additional loxP site 5′ of exon 5. As a result, the 3′ *Bsr*gI and the 5′ *Bcl*I restriction site were destroyed, as indicated by red crosses. After electroporation of the construct in ES cells, $G418^r$ $Ganc^r$ clones were screened by southern blot hybridization for homologous recombination events (*Bsr*gI/*Bgl*I digest, probe 1, wt allele: 4.6 kb, targeted allele: 9.3 kb; *Xba*I digest, probe3, wt allele: 6 kb, targeted allele: 9 kb) (data not shown). Clones containing the targeted allele were expanded and *Cre* recombinase was transiently expressed in those cells. Clones non-resistant to G418 were screened by southern blot (*Bsr*gI/*Bgl*I digest, probe1, wt allele 4.6 kb, targeted allele 6.3 kb) for loss of the Neo cassette and presence of the floxed exon 5. Mice heterozygous for the targeted *Fgfr2* allele were generated using standard procedures. After tissue-specific expression of the *Cre* recombinase, recombination between the two loxP sites flanking exon 5 takes place resulting in deletion of exon 5. Mice are genotyped either by PCR (primers are indicated as blue triangles, wt band 0.8 kb, floxed band 1.1 kb) (A′) or by southern blotting (A″) using probe 2 after *Bgl*II digest. The wt allele is 2.4 kb, the floxed allele 2.6 kb. Ig: Ig-like domain; a: acidic box; TM: transmembrane domain; TK: tyrosine kinase domain; 4: exon 4; 5: exon 5; 6: exon 6; Bc: *Bcl*I; Bg1: *Bgl*I; Bg2: *Bgl*II; Bs: *Bsr*gI; RI: *Eco*RI; X: *Xba*I. (B) *Fgfr2* exon 5 is not expressed in the embryonic mid-/hindbrain region (MHR) after recombination. Radioactive *in situ* hybridizations (*ISHs*) were performed on consecutive sagittal sections of an E9 $En1^{Cre/+}$ $Fgfr2^{lox/lox}$ (b–d) and wt littermate (a) mouse embryo. *Fgfr2* (*Fgfr2* exon 5 probe) is expressed throughout the MHR (a). *Cre* recombinase expression is detected in the presumptive MHR (midbrain and r1) (b). *Fgfr2* exon 5 expression is lost specifically in the MHR (c). The false-color overlay of the *Fgfr2* exon 5 expression (red) and the *Cre* recombinase expression (green) in the MHR demonstrates that the gap in *Fgfr2* exon 5 expression corresponds to the *Cre* recombinase expression domain in the MHR (d). The dorsal and ventral MHB is marked by red arrowheads. hb: hindbrain; mb: midbrain. Scale bar = 500 μm. (C) Deletion of *Fgfr2* exon 5 in genomic DNA from the MHR of the adult $En1^{Cre/+}$ $Fgfr2^{lox/lox}$ mouse. PCR was performed using the genotyping primers on different tissues of the MHR. The mutant allele (0.6 kb) was detected in the inferior and superior colliculi (IC, SC) as well as in the cerebellum (CB) of the homozygous $En1^{Cre/+}$ $Fgfr2^{lox/lox}$ mice. In contrast, the control tissues cortex (Cx), olfactory bulbs (OB) and the tail displayed the floxed allele (1.1 kb). PCR on cerebellum of control animals $En1^{+/+}$ $Fgfr2^{lox/lox}$ also resulted in the floxed allele (ctrl-CB).

Yaylaoglu et al. (2005). *GAD65*, *Sert*, *TH* and *PLP20* probes were DIG-labeled according to this protocol.

Histochemistry and immunohistochemistry

Nissl staining was performed with a 0.5% Cresyl violet solution according to standard procedures.

Immunohistochemistry on paraffin sections was performed as described before (Brodski et al., 2003) with antibodies directed against parvalbumin (rat anti-parvalbumin, 1:500; Swant).

Results

Generation of a conditional Fgfr2 allele

To study the role of the Fgfr2 in the development of the mid-/hindbrain region, a conditional *Fgfr2* KO mouse was generated. In the targeting construct, a loxP site was introduced 3′ of Fgfr2 exon 5. Additionally, a TK neo cassette, which itself was flanked by two loxP sites and was introduced 5′ of exon 5, was

utilized for selection of the recombination event in ES cells but was subsequentially removed by transient *Cre* expression in ES cells. After testing of the ES cells for the loss of the neo cassette and presence of the floxed exon 5, cells were used to generate the mutant mouse *Fgfr2*$^{lox/lox}$ (Fig. 1A). Deletion of exon 5 of the *Fgfr2*, which encodes the first part of the Ig-like domain II, results in a stop codon in the extracellular domain within exon 6, which disrupts translation before the extracellular ligand binding Ig-like III domain. Therefore, the protein translated from the exon 5 deleted *Fgfr2* transcript, if any, would be non-functional.

Mice hetero- or homozygous for the *Fgfr2*$^{lox/lox}$ allele were phenotypically indistinguishable from their wild-type littermates. Thus, the introduced loxP sites themselves do not appear to interfere with *Fgfr2* expression (Figs. 1B, a). To test the functionality of the *Fgfr2*$^{lox/lox}$ allele, we crossed the *Fgfr2*$^{lox/lox}$ mice with mice carrying a Pgk-Cre transgene driving ubiquitous *Cre* expression (Lallemand et al., 1998). Embryos homozygous for the *Fgfr2*$^{lox/lox}$ allele showed early death at around E5, closely resembling the *Fgfr2*-null mutants reported previously (Arman et al., 1998; data not shown). Therefore, *Fgfr2*$^{lox/lox}$ behaves as a conditional allele, which can be fully inactivated by tissue-specific *Cre* recombinase expression.

In parallel, we used an additional floxed *Fgfr2* mouse line, which has been published before (Yu et al., 2003). In this mouse, exons 7, 8 and 9 were targeted. Deletion of these exons within the *Dermo1* as well as the *β-actin* expression domains resulted in obvious phenotypes, which proved that this conditional mouse is functional as well. Both floxed *Fgfr2* mouse lines used in the present analysis showed the same unaltered mid-/hindbrain region phenotype at stages E12.5 and E18.5, and hence the presented data are based on the *Fgfr2*$^{lox/lox}$ mouse line in which the *Fgfr2* exon 5 is floxed.

Tissue-specific inactivation of the Fgfr2 in the mid-/hindbrain region

To study the role of the Fgfr2 in the transduction of Fgf signals in the mid-/hindbrain region and therefore its role in the patterning activity of the MHO, the *Fgfr2* was inactivated in the midbrain and anterior hindbrain. For this purpose, we used the *En1*$^{Cre/+}$ mouse, which expresses the *Cre* recombinase from the *En1* locus (Kimmel et al., 2000; Chi et al., 2003). *En1* expression starts at the 2-somite stage. After the initial regionalization of the midbrain and hindbrain (E8), *En1* expression is spanning the midbrain and anterior hindbrain (r1). To analyze the pattern of *Cre* recombinase expression and therefore inactivation of the *Fgfr2* exon 5, radioactive *in situ* hybridization was performed using an exon 5-specific probe. Reduction of the signal was observed already at the 8-somite stage (data not shown), and at the 15-somite stage (E9) the mid-/hindbrain region was negative for the expression of exon 5 when compared to the expression of exon 5 in the wild-type situation (Fig. 1B). These results are in congruence with published data on the functionality of the *En1*$^{Cre/+}$ mouse line (Trokovic et al., 2003; Chi et al., 2003; Li et al., 2002). Furthermore, in the adult mutant mouse loss of the *Fgfr2* exon 5 was assessed using a PCR on genomic DNA isolated from different brain regions. Deletion of exon 5 was specific for the mid-/hindbrain region (Fig. 1C). Thus, we conclude that *Fgfr2* in the mid-/hindbrain region is inactivated from the 8-somite stage onward and therefore is unable to transmit the Fgf signal at the MHO.

Normal patterning of the mid-/hindbrain region during embryogenesis in the En1$^{Cre/+}$ *Fgfr2*$^{lox/lox}$ *mouse*

Fgf8 was shown to ectopically regulate the expression of other MHO genes (Crossley et al., 1996; Martinez et al., 1999; Liu et al., 1999; Liu and Joyner, 2001b). The early loss of the *Fgfr2* in the mid-/hindbrain region was expected to result in an impairment of Fgf8 signaling at the MHO, leading to changes in MHO gene expression. Therefore, we analyzed the expression of the genes *En1*, *Pax2*, *Otx2* and *Gbx2* and *Fgf8* itself in the A/P axis and of *Shh* and *Wnt1* in the D/V axis at E10.5 and E12.5 (Fig. 2A and data not shown), when the genetic cascade of the MHO is fully established and changes in gene expression should be visible. An impairment of Fgf8 signaling should lead to a down-regulation of *Gbx2*, *En1*, *Wnt1* and an up-regulation of *Otx2*, which was shown to be the case in the dorsal mid-/hindbrain region of the *Fgfr1* conditional KO and in the conditional *Fgf8* KO (Trokovic et al., 2003; Chi et al., 2003). Interestingly, none of these genes were altered in their pattern of expression. Even in earlier E9.5 embryos, in which both alleles of *Fgfr2* and also one allele of *Fgfr1* had been inactivated (using intercrosses with the conditional Fgfr1 knockout line; Trokovic et al., 2003), no changes in early expression of *Otx2*, *Gbx2*, *En1*, *Fgf8* and of *Erm*, a direct target of Fgf signaling, were found (Supplementary Fig. 1), indicating that the *Fgfr2* does not lead to temporary changes concerning the patterning of the mid-/hindbrain region.

To evaluate the histological integrity of the developing mid-/hindbrain region, in particular of its ventral parts, the expression of genes marking distinct territories in this region was examined. *TH* marks catecholaminergic neurons located in the floor and basal plate of the ventral midbrain and herein marking the dopaminergic neurons. The most ventral *Nkx2.2*-positive population marks the basal plate/alar plate boundary, *Nkx6.1* expression marks a not yet fully characterized subpopulation of motoneurons and neurons of the red nucleus (RN), a motor nucleus in the rostral midbrain. *Pou4f1* expression labels all the prospective neurons of the RN, which are located in the basal plate of the midbrain. These marker genes were unaltered in the mutant embryos, revealing that histology is normal in the developing mid-/hindbrain region (Fig. 2B).

Neuronal subpopulations and oligodendrocytes develop a normal phenotype in the En1$^{Cre/+}$ *Fgfr2*$^{lox/lox}$ *mouse and are maintained in the mid-/hindbrain region of the adult En1*$^{Cre/+}$ *Fgfr2*$^{lox/lox}$ *mouse*

Although the Fgfr2 seems to be dispensable for the transmission of the patterning effect of Fgf8 signaling at the MHO, it is possible that it plays a role in the development of specific

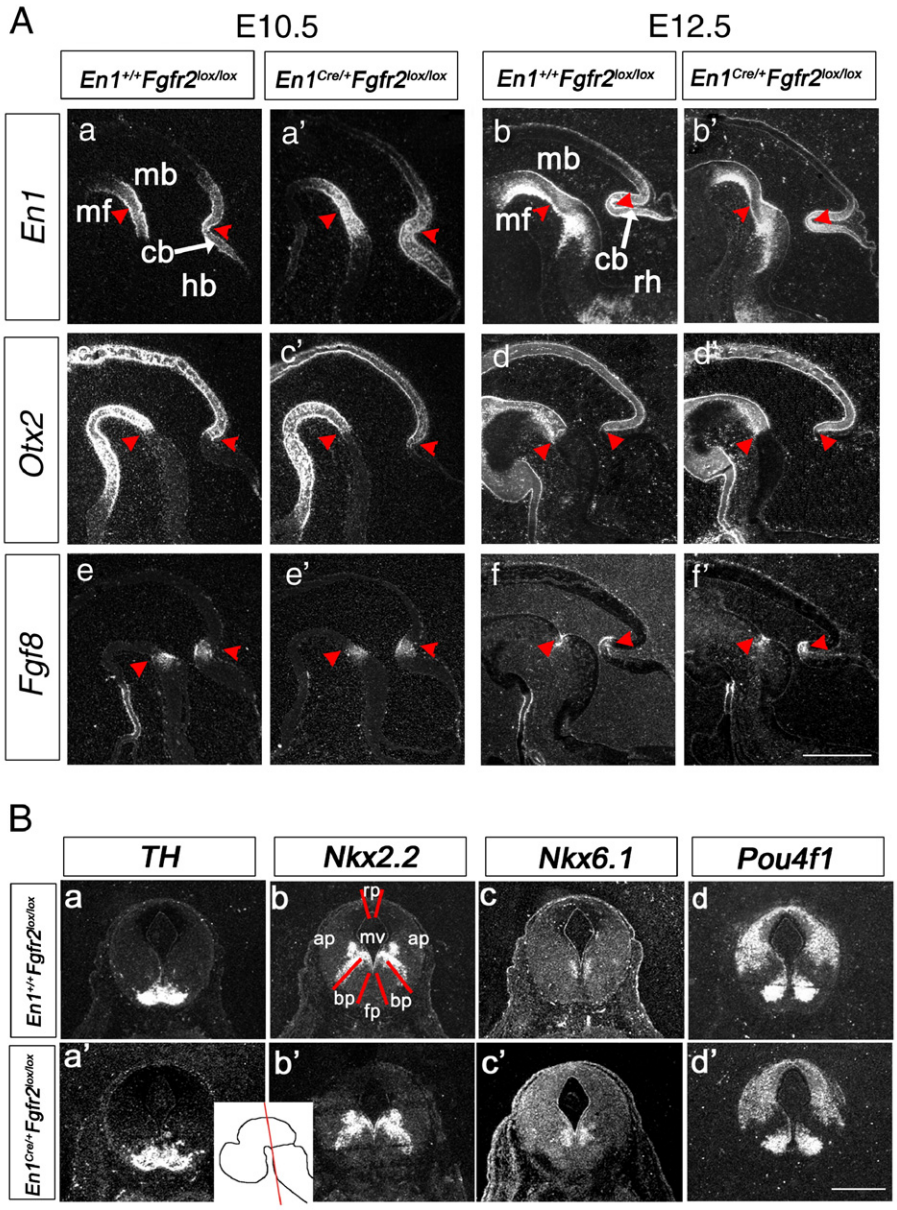

Fig. 2. Normal patterning and histology of the MHR in embryogenesis of the $En1^{Cre/+}$ $Fgfr2^{lox/lox}$ mouse. Radioactive *in situ* hybridizations were performed on sagittal sections of E10.5 and E12.5 (A) and consecutive coronal sections of E12.5 (B) $En1^{Cre/+}$ $Fgfr2^{lox/lox}$ mouse embryos. Close-ups of the MHR (A) or the neural tube at the level of the midbrain (B) are shown. (A) *En1* is expressed in the presumptive MHR at E10.5 and its expression shows no difference in the $En1^{Cre/+}$ $Fgfr2^{lox/lox}$ mouse (a, a′). At E12.5 *En1* expression marks the posterior midbrain and r1. Also at this time point expression of *En1* is unchanged in the mutant (b, b′). Both at E10.5 and E12.5 *Otx2* is expressed in the midbrain up to the mid-/hindbrain boundary (MHB). No difference in expression can be seen in the mutant mouse (c, c′, d, d′). *Fgf8* expression at E10.5 and E12.5 is localized in the anterior part of r1 and is unchanged in the $En1^{Cre/+}$ $Fgfr2^{lox/lox}$ mouse (e, e′, f, f′). The dorsal and ventral MHB are marked by red arrowheads. Cb: cerebellar Anlage; hb: hindbrain; mb: midbrain; mf: mesencephalic flexure; rh: rhombencepalon. (B) At E12.5 *TH* is expressed in the floor plate and basal plate and marks the future dopaminergic neurons of the midbrain. Its expression is not changed in the mutant mouse (a, a′). *Nkx2.2* is expressed at the alar/basal plate boundary and in an additional small ventricular population in the alar plate. Its expression is not changed in the mutant mouse (b, b′). *Nkx6.1* is expressed in the basal plate of the midbrain where it marks a subpopulation of the red nucleus and motoneurons and its expression is also not changed in the mutant mouse (c, c′). *Pou4f1* is expressed almost throughout the whole neural tube with exception of the alar/basal plate boundary and in the basal plate where it marks the red nucleus. There is no difference in the expression domain of *Pou4f1* in the control and mutant mouse (d, d′). The insert in a′/b′ shows the plane of sectioning. ap: alar plate; bp: basal plate; Fp: floor plate; mv: mesencephalic vesicle; rp: roof plate. Scale bar = 500 μm.

neuronal populations during embryogenesis and – due to its sustained expression into adulthood (own unpublished data and Belluardo et al., 1997) – also in the maintenance of tissue and certain neuronal populations in the mid-/hindbrain region.

To examine the role of Fgfr2 in the development of specific neuronal subpopulations of the mid-/hindbrain region, we analyzed in E12.5 embryos the expression pattern of *TH* as a marker for catecholaminergic neurons in the ventral midbrain (future dopaminergic neurons) as well as for noradrenergic

neurons in r1, which will become the future locus caeruleus (LC). Furthermore, we used *Sert* as a marker for serotonergic (5-HT) neurons (the dorsal raphe 5-HT neurons arise from r1), *GAD65* for the GABAergic neurons of the mid-/hindbrain region and *Islet1* for motoneurons in this region (future 3rd and 4th cranial nerve nuclei). The expression of these genes was unchanged and revealed that neuronal subpopulations develop without any impairment in the mutant mice (Fig. 3A).

Next, we analyzed the function of Fgfr2 in the general maintenance of adult tissue in the mid-/hindbrain region. As

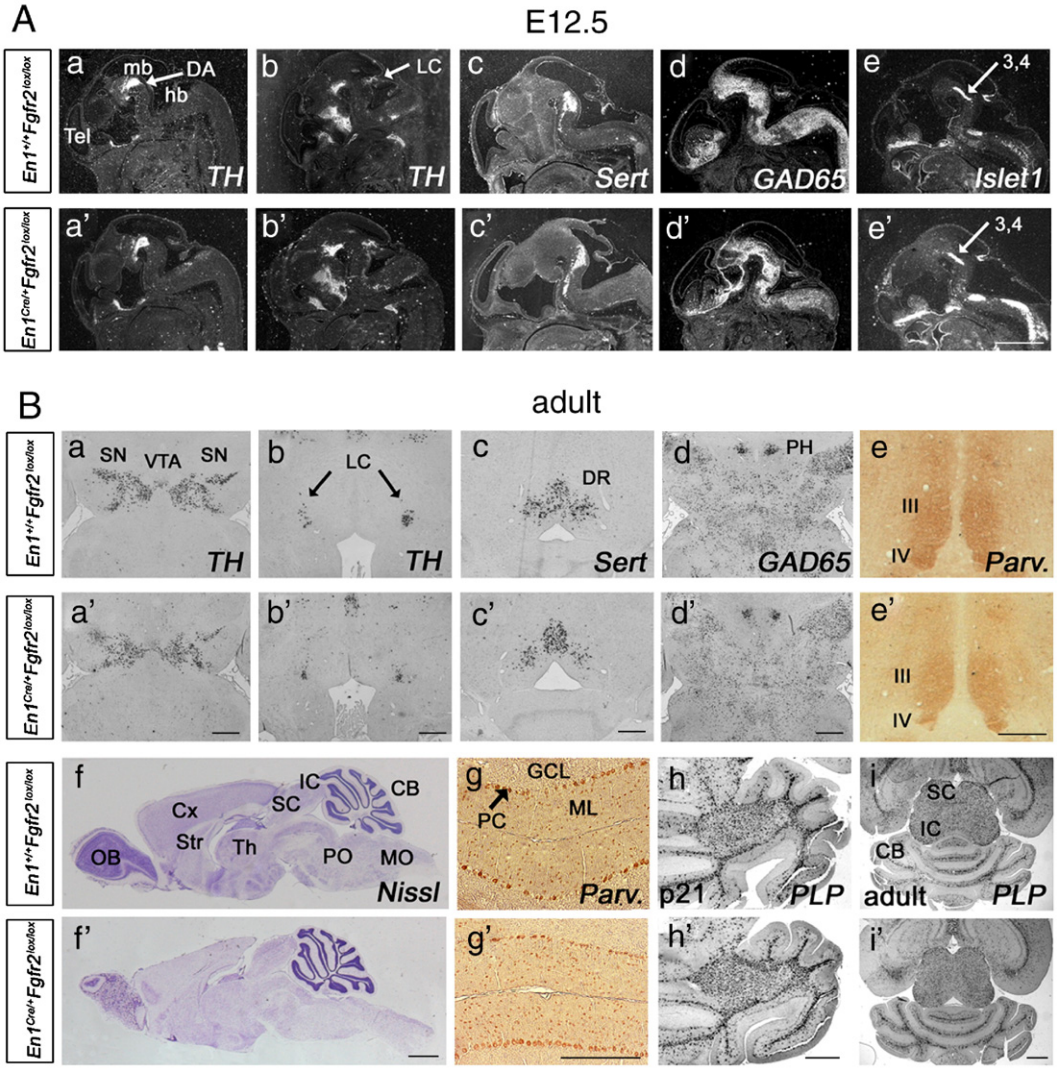

Fig. 3. Neuronal subpopulations develop normal in the *En1*$^{Cre/+}$ *Fgfr2*$^{lox/lox}$ mouse and are maintained in the MHR of the adult *En1*$^{Cre/+}$ *Fgfr2*$^{lox/lox}$ mouse. The general morphology of the MHR of the mutant mice is unchanged. Oligodendrocytes of p21 and adult brain are not altered. Radioactive *in situ* hybridizations were performed on sagittal sections of E12.5 *En1*$^{Cre/+}$ *Fgfr2*$^{lox/lox}$ mouse embryos. Close-ups of the anterior neural tube are shown (A). With respect to the MHR, *TH* is expressed in the ventral midbrain, where it marks the presumptive dopaminergic neurons of the midbrain and in the presumptive locus caeruleus (ventral r1) (a, b). *TH* expression in both regions of the MHR is unchanged in the mutant mice (a, a′, b, b′). Serotonergic (5-HT) neurons, visualized by the expression of the serotonin transporter *Sert*, develop normally in the dorsal raphe nuclei and are present in the control as well as in the mutant mice (c, c′). Terminally differentiated GABAergic neurons of the MHR are also unchanged in the MHR of the mutant mice as revealed by the *GAD65* ISH probe (d, d′). The motor neurons of the cranial nerve nuclei developing in the MHR (3rd and 4th cranial nerve) are located in the same position in the control as well as in the mutant mice (e, e′). 3: 3rd cranial nerve motor nucleus; 4: 4th crainial nerve motor nucleus; DA: dopaminergic neurons of mb; hb: hindbrain; LC: locus nucleus; mb: midbrain; Tel: telencephalon. (B) Non-radioactive *in situ* hybridizations were performed on adult horizontal sections of control and mutant mice. Dopaminergic neurons of the substantia nigra (SN) and ventral tegmental area (VTA) are present in the correct location and number in control and mutant mice, as determined by the use of the *TH ISH* probe (a, a′). Also the noradrenergic neurons of the LC are comparable in the control and mutant (b, b′). Serotonergic neurons are also unchanged in the mutant as revealed by Sert expression (c, c′). GABAergic neurons in the MHR (here at the same level where the DA neurons of the SN and VTA were assessed) were also present (d, d′). The cranial nerves of the MHR (3rd and 4th) were present and in the right location in the mutant mice as determined by Parvalbumin immunohistochemistry (e, e′). The general morphology of the MHR, shown by Nissl staining, is unchanged in the mutant mice compared to the control animals (f, f′). Furthermore, the Purkinje cells of the cerebellum are normal in size and distribution (g, g′). Oligodendrocytes are present in the MHR in comparable numbers at p21 (here only the cerebellum is shown) as well as in adulthood (h, h′, i, i′). SN: substantia nigra; VTA: ventral tegmental area; LC: locus caeruleus; DR: dorsal raphe nuclei; PH: posterior hypothalamic area; III: 3rd cranial nerve; IV: 4th cranial nerve; Cx: cortex; OB: olfactory bulb; Th: thalamus; CB: cerebellum; PO: pons; MO: medulla oblongata; IC: inferior colliculi; SC: superior colliculi; Str: striatum; GCL: granule cell layer; PC: Purkinje cell layer; ML: molecular layer. Scale bars = 500 μm (f, f′) and 1 mm (h, h′, i, i′).

revealed by Nissl staining and Parvalbumin immunohistochemistry, the general brain morphology was normal and the histological integrity of the mid-/hindbrain region was unaltered when compared to littermate controls. In particular, the cerebellum showed a normal foliation pattern and the Purkinje cells, which strongly express the *Fgfr2* in the adult mouse (data not shown), were present and appeared normal. Furthermore, the third and fourth cranial nerve nuclei, which reside in the mid-/hindbrain region, were present and undistinguishable from the littermate control (Figs. 3B, e–g′).

In order to determine whether *Fgfr2* has a specific role in the maintenance of neuronal subpopulations in adults, we analyzed the mutant mice with respect to the distribution of the catecholaminergic neuron marker *TH* (which also in adults marks the dopaminergic neurons of the substantia nigra (SN) and ventral tegmental area (VTA) and in the hindbrain the noradrenergic neurons of the LC), *Sert* as marker for 5-HT neurons and *GAD65* as marker for GABAergic neurons in the mid-/hindbrain region. Using a non-radioactive *in situ* hybridization method on brain sections of adult mutants and their control littermates, no obvious qualitative changes in the distribution and number of cell bodies of these neuronal subpopulations were detected (Figs. 3B, a–d′).

We also analyzed the presence of terminally differentiated oligodendrocytes in the mid-/hindbrain region, as the *Fgfr2* is strongly expressed in fiber tracts of the mid-/hindbrain region in mouse and rat, in particular in the superior and inferior colliculi (Asai et al., 1993; own data not shown) and in glia cells (Yazaki et al., 1994; Asai et al., 1993, Bansal et al., 2003). *Fgfr2* expression is also found in the ventral midbrain where oligodendrocyte precursor cells arise (Fu et al., 2003; Blak et al., 2005). Due to these expression patterns, we hypothesized that *Fgfr2* might be involved in the differentiation and/or maintenance of oligodendrocytes. The expression of the terminal marker for oligodendrocytes *PLP20* was unchanged at p21 and in adulthood, revealing that the Fgfr2 is dispensable for the terminal differentiation and maintenance of oligodendrocytes in the mid-/hindbrain region. (Figs. 3B, h–i′).

Taken together, these data indicate that Fgfr2 alone is dispensable for the patterning process at the MHO as revealed by the unchanged MHO gene expression as well as the histological integrity of the mid-/hindbrain region in the mutant $En1^{Cre/+}$ $Fgfr2^{lox/lox}$ mice. Furthermore, catecholaminergic, GABAergic and serotonergic neurons were not affected, which shows that the *Fgfr2* per se is not essential for the development and maintenance of specific neuronal populations in the mid-/hindbrain region. As the Fgfr2 is not necessary for Fgf8 signal transduction in the mid-/hindbrain region, we speculated that – even though *Fgfr3* expression displays a bigger gap in the mid-/hindbrain region in embryogenesis – the Fgfr3 might be involved in signal transduction in the ventral mid-/hindbrain region. Therefore, we analyzed the *Fgfr3* KO mice ($Fgfr3^{-/-}$) to determine the role of the Fgfr3 in the ventral mid-/hindbrain region.

$Fgfr3^{-/-}$ mutant mice

The $Fgfr3^{-/-}$ mutant mouse was described previously (Colvin et al., 1996). A brain phenotype has not been found for a long time. Recently, the Fgfr3 has been implicated in the terminal differentiation of oligodendrocytes (Oh et al., 2003) and in the control of proliferation and apoptosis of cortical progenitors (Inglis-Broadgate et al., 2005).

Patterning of the mid-/hindbrain region is normal in the $Fgfr3^{-/-}$ mouse

In analogy to the analysis of the conditional *Fgfr2* mouse, the analysis of the $Fgfr3^{-/-}$ mice focused on the mid-/hindbrain region. First, the expression of MHO genes, which were shown to be ectopically regulated by Fgf8 (see above), was examined. As in the $En1^{Cre/+}Fgfr2^{lox/lox}$ mouse, MHO genes were not altered in their expression pattern in the $Fgfr3^{-/-}$ mice (Fig. 4A and data not shown). Furthermore, whole-mount *in situ* analysis at E9.5 revealed no temporary alterations in patterning or Fgf signaling (Supplementary Fig. 1).

Brain morphology and neurotransmitter systems are not changed in the adult $Fgfr3^{-/-}$ mouse

In order to examine the role of the Fgfr3 in maintenance of the mid-/hindbrain region and neural populations therein, the histological integrity of the tissue and the presence of neuronal populations was analyzed in the adult $Fgfr3^{-/-}$ mouse. Indeed, the *Fgfr3* is expressed in the adult mid-/hindbrain region (Belluardo et al., 1997). Furthermore, postnatal *Fgfr3* expression increases from P2 until P9 (Oh et al., 2003). This increase could well correlate with a role for Fgfr3 in the maintenance of neural populations in late embryogenesis and adulthood. Utilizing Nissl staining and immunohistochemistry for Parvalbumin, we found a histologically unaltered mid-/hindbrain region with the 3rd and 4th cranial nerve nuclei being present (Figs. 4B, d–e′ and h, h′). Furthermore, the mutant mice showed no apparent abnormality in *GAD65*, *TH* and *Sert* expression, revealing that the cell bodies of GABAergic, catecholaminergic and 5-HT neurons were present and normally distributed in the mid-/hindbrain region, compared to littermate controls (Figs. 4B, a–c′). It has been shown before that oligodendrocyte differentiation is delayed in $Fgfr3^{-/-}$ mice. Terminally differentiated oligodendrocytes reach their normal numbers at p31 (Oh et al., 2003). Analysis of PLP expression in adult $Fgfr3^{-/-}$ mice did not reveal any obvious changes in the mutants (Figs. 4B, f–g′). Since we analyzed older mice, we showed that the Fgfr3 does not play a role in maintenance of these cells in adulthood.

This suggests that in analogy with the data obtained from the analysis of the $En1^{Cre/+}$ $Fgfr2^{lox/lox}$ mice, also the inactivation of the *Fgfr3* does neither have an effect on the early patterning function of Fgf8 at the MHO nor does it impair the formation and maintenance of neuronal populations in the mid-/hindbrain region.

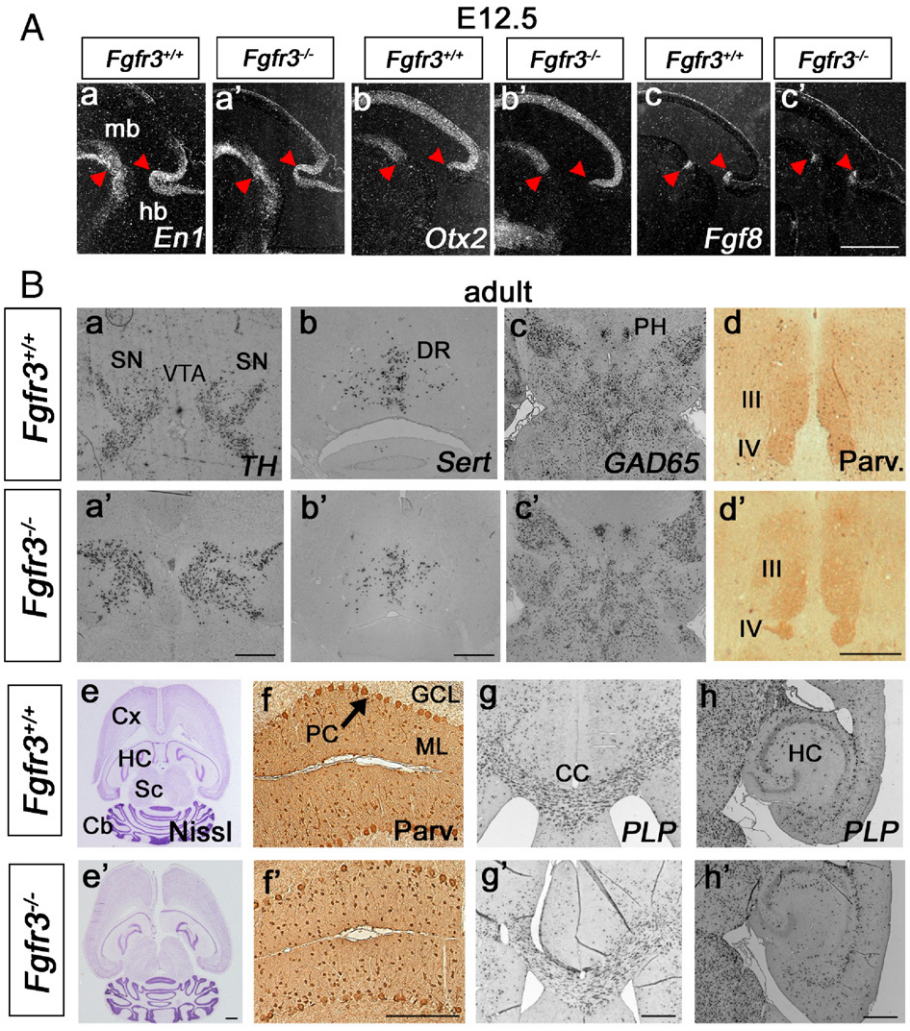

Fig. 4. The *Fgfr3*$^{-/-}$ mouse reveals normal patterning of the MHR during embryogenesis. Neuronal subpopulations are maintained in the MHR of the adult *Fgfr3*$^{-/-}$ mouse. The general morphology of the MHR as well as oligodendrocytes are unchanged in the mutant mice. Radioactive *in situ* hybridizations were performed on sagittal sections of E12.5 *Fgfr3*$^{-/-}$ mouse embryos. Close-ups of the MHR are shown (A). *En1* is expressed in the presumptive MHR at E12.5 and its expression shows no difference in localization in the *Fgfr3*$^{-/-}$ mouse (a, a′). *Otx2* is expressed in the midbrain up to the mid-/hindbrain boundary (MHB). No difference in expression can be seen in the mutant mouse (b, b′). *Fgf8* expression is confined to anterior r1. Comparable expression can be seen in the *Fgfr3*$^{-/-}$ mouse (c, c′). hb: hindbrain; mb: midbrain. (B) Non-radioactive *in situ* hybridizations were performed on adult horizontal sections of control and mutant mice. Dopaminergic neurons of the substantia nigra (SN) and ventral tegmental area (VTA) are present in the right place and number in the control and mutant mice as determined by the use of the *TH ISH* probe (a, a). Serotonergic neurons are present in the dorsal raphe nuclei of the control as well as of the mutant mice as shown by *Sert* expression (b, b′). GABAergic neurons of the MHR are unchanged in the MHR of the adult mutant mice as revealed by the *GAD65 ISH* probe (c, c′). Parvalbumin staining shows that the 3rd and 4th cranial nerve are present in the adult mutant mice (d, d′). The general morphology of the MHR, as determined by Nissl staining, is unchanged in the mutant mice compared to the control animals (e, e′). Also the Purkinje cells of the cerebellum are normal in size and distribution (f, f′). Oligodendrocytes are present in the brain in normal numbers in adulthood (g, g′, h, h′). SN: substantia nigra; VTA: ventral tegmental area; DR: dorsal raphe nuclei; PH: posterior hypothalamic area; III: 3rd cranial nerve; IV: 4th cranial nerve; Cx: cortex; HC: hippocampus; Sc: superior colliculi; Cb: cerebellum; CC: corpus callosum. Scale bars = 500 μm (e) and 1 mm (e′).

No alterations in the Fgf signaling cascade at the mid-/hindbrain boundary of the mutant En1$^{Cre/+}$ *Fgfr2*$^{lox/lox}$ *mice and Fgfr3*$^{-/-}$ *KO mice*

Since we could not detect any major changes in neither the conditional Fgfr2 knockout nor in the Fgfr3 knockout, we were interested whether – despite the loss of functional Fgf receptors – this may be attributed to an unaltered downstream Fgf signaling cascade.

Several downstream components are activated by Fgf signaling at the MHO: the RAS/MAPK signaling pathway, the phosphoinositol-3 kinase/AKT (PI3K/AKT) pathway and the phospholipase Cγ/protein kinase C (PLCγ/PKC) pathway. *Erm*, *Pea3*, *Mkp3*, *Sprouty1* (*Spry1*) and *Sef1* genes are downstream targets of Fgfs, with the latter three being negative modulators of Fgf signaling (Niehrs and Meinhardt, 2002; Tsang and Dawid, 2004; Echevarria et al., 2005). As suggested by the above-presented results, we indeed were unable to detect any differences in the expression of *Mkp3*, *Erm*, *Spry1* or *Sef1* (Fig. 5). Furthermore, we could exclude a possible compensatory up-regulation of the other Fgfrs, in particular of *Fgfr1* and *3* in the conditional *Fgfr2* KO mouse (Fig. 5) and of the *Fgfr1*

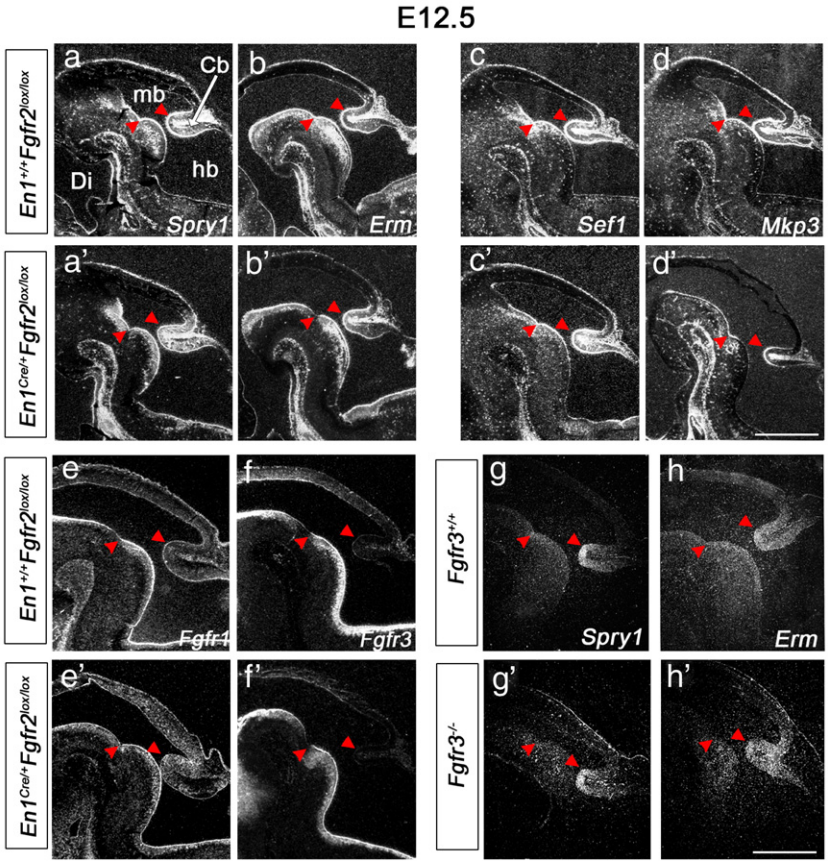

Fig. 5. The downstream signaling cascade is not altered at the MHB of mutant $En1^{Cre/+}$ $Fgfr2^{lox/lox}$ mice and $Fgfr3^{-/-}$ KO mice. Radioactive *in situ* hybridizations were performed on sagittal sections of E12.5 $En1^{Cre/+}$ $Fgfr2^{lox/lox}$ and $Fgfr3^{-/-}$ mouse embryos. Close-ups of the MHR are shown. *Spry1*, *Erm*, *Sef1* and *Mkp3* are expressed ventrally along the entire MHR and dorsally in the IC and the cerebellar anlage. Their expression patterns are unchanged in the $En1^{Cre/+}Fgfr2^{lox/lox}$ mice (a, a′; b, b′; c, c′; d, d′). The *Fgfr1* is expressed along the ventral and dorsal MHR and is unchanged in the mutant $En1^{Cre/+}$ $Fgfr2^{lox/lox}$ mouse (e, e′). The *Fgfr3* is ventrally expressed in the anterior midbrain and in entire r1, leaving a gap in the caudal midbrain, whereas dorsally its expression displays a bigger gap in the MHR. This characteristic expression pattern is unchanged in the mutant $En1^{Cre/+}$ $Fgfr2^{lox/lox}$ animals (f, f′). In $Fgfr3^{-/-}$ mutant mice, expression of *Spry1* and *Erm* is also unchanged as compared to the wild-type control (g, g′; h, h′). Red arrowheads mark the ventral and dorsal MHB. Di: diencephalon; mb: midbrain; hb: hindbrain. Scale bar = 500 μm.

and *2* in the *Fgfr3* KO mouse (data not shown). Other members of the Fgf family, *Fgf15*, *17* and *18*, were also not altered on the transcriptional level (data not shown).

Taken together, the analysis of the components of the Fgf signaling pathway at the mid-/hindbrain boundary did not reveal any changes in the $En1^{Cre/+}$ $Fgfr2^{lox/lox}$ and the $Fgfr3^{-/-}$ mouse.

Discussion

The conditional Fgfr2 mutant and the Fgfr3 knockout show no obvious phenotype in the mid-/hindbrain region

The activity of the MHO, which is characterized by a tightly regulated network of genes, has been shown to be essential for the correct patterning of the prospective midbrain and anterior hindbrain. Bead transplantation experiments and electroporation experiments in chicken have demonstrated that the “b” isoform of Fgf8 is able to regulate the expression of several MHO genes and mimic the patterning function of the MHO (Crossley et al., 1996; Martinez et al., 1999; Shamim et al., 1999; Liu et al., 1999; Liu and Joyner, 2001b). But so far the Fgfrs responsible for the transmission of the Fgf8b signal have only been partly identified. The conditional knockout of the *Fgfr1* in the mid-/hindbrain region showed a dorsal patterning defect in this region, resulting in a lack of the inferior colliculi of the midbrain and the vermis of the anterior hindbrain. In contrast to the loss of dorsal structures, the ventral mid- and hindbrain regions are largely unaffected. Interestingly, this phenotype does not recapitulate the phenotype of the conditional *Fgf8* KO mouse, where indeed dorsal as well as ventral parts of the mid-/hindbrain region are missing. Therefore, it is highly likely that the Fgfr1 is not the only receptor capable to transduce the Fgf8 signal at the MHO, especially in the ventral mid-/hindbrain region.

Recently, we could show that also *Fgfr2* and *Fgfr3* are expressed in specific territories of the ventral mid-/hindbrain region during the establishment and refinement of the MHO (from E8.5 on). Furthermore, expression of *Fgfr2* and *Fgfr3* overlaps with the expression of *Spry1*, a target gene of Fgf signaling (Trokovic et al., 2005). This suggests that one or even both of these receptors may be involved in the patterning

function of Fgf8 in this region. Therefore, in order to determine the function of the *Fgfr2* and *Fgfr3* in the ventral mid-/hindbrain region, we have analyzed mouse mutants lacking either *Fgfr2* or *Fgfr3* in the mid-/hindbrain region. Conventional loss-of-function of the *Fgfr2* results in a very early lethal phenotype due to a failure in gastrulation (Arman et al., 1998), whereas knockout mice for either one of the two isoforms, *Fgfr2IIIb* or *Fgfr2IIIc*, as well as the hypomorphic mouse mutant of the *Fgfr2*, in which the IgIII domain is deleted, were not analyzed in respect to a brain phenotype (Xu et al., 1998; De Moerlooze et al., 2000; Revest et al., 2001a,b; Eswarakumar et al., 2002). In the *Fgfr3* mutant mice, defects in the terminal differentiation of oligodendrocytes and in the development of cortical progenitors were found recently (Oh et al., 2003; Inglis-Broadgate et al., 2005). However, the effect of Fgfr3 deficiency on the mid-/hindbrain region was not described. Here we presented the first detailed analysis of the mid-/hindbrain region in mice exhibiting a loss in Fgfr2 or Fgfr3 function in this region. The thorough analysis of the mutant mice showed clearly that both receptors alone are dispensable for the formation of a normal phenotype and maintenance of the mid-/hindbrain region, since we have not found any alterations in the expression of early patterning genes nor in the embryonic and adult histological or cellular integrity in this region.

In a recent study, it has been shown that Fgf receptors are instrumental for the maintenance of dopaminergic neurons in the adult substantia nigra (Corso et al., 2005). Our results provide evidence that Fgfr2 and Fgfr3 on their own do not play a role in the maintenance of the neuronal populations studied in our analyses, including the substantia nigra dopaminergic neurons.

We cannot exclude, however, that the Fgfr2 and Fgfr3 have specific functions during development and in the adult brain, which after deletion of the gene in the mid-/hindbrain region could lead to subtle effects that could not be detected in our analyses. For example, both Fgfrs could play a role in neurite outgrowth, axonal extension and ramification as was shown to be a property of Fgfrs in general (Bulow et al., 2004; for a review, see Reuss and von Bohlen und Halbach, 2003; McFarlane et al., 1996; Doherty and Walsh, 1996; Williams et al., 1994; Saffell et al., 1997; Niethammer et al., 2002). However, to detect such changes in vivo additional morphological analysis is required.

Functional redundancy between the Fgfrs 1, 2 and 3

Because of the intact Fgf signaling pathway in the conditional *Fgfr2* and the *Fgfr3* KO mice, the most likely explanation for the unchanged phenotype of the ventral mid-/hindbrain region in both analyzed mutant mouse lines is a functional redundancy between the Fgfrs 1, 2 and/or 3 in this region. The expression of the Fgf receptors 1–3 in the mid-/hindbrain region shows substantial overlap, which supports a possible redundancy of these receptors in the development of this region. Also the Fgfr4 has to be taken under consideration to act in the mid-/hindbrain region, although its expression in this region is still under discussion (Stark et al., 1991; Miyake et al., 1995; Ozawa et al., 1996; Yaylaoglu et al., 2005; Blak et al., 2005).

Redundancy between the Fgfrs concerning their function in the development of the mid-/hindbrain region is also supported by a publication in which activated Fgfr1 and Fgfr3 have both been shown to be able to induce En2 when expressed ectopically in anterior midbrain and posterior diencephalon (Kobayashi et al., 2002). Furthermore, in the forebrain, Fgfr1 conditional knockouts show defects in the olfactory bulbs (Hebert et al., 2003), while a conditional single knockout for *Fgfr2* and the *Fgfr3* knockout do not show patterning defects in the telencephalon (Gutin et al., 2006). However, double knockouts for *Fgfr1* and either *Fgfr2* or *Fgfr3* in the telencephalon show severe defects in development of the ventral telencephalon (Gutin et al., 2006), suggesting redundancy of Fgf receptors in telencephalic development. In contrast to a functional redundancy of the Fgf receptors in transduction of the MHO activity or in telencephalic patterning, in other embryonic processes a redundant function of the Fgfrs has been excluded, either due to distinct expression patterns (e.g. during gastrulation and somitogenesis; Orr-Urtreger et al., 1991; Yamaguchi et al., 1991; Walshe and Mason, 2000) or as was recently shown in the developing tail-bud and pharyngeal arches, by the fact that impaired Fgfr1 signaling cannot be rescued by the presence of the co-expressed Fgfr2 (Hoch and Soriano, 2006). These differences between regions and developmental processes, however, clearly point towards a context-specific requirement for Fgfr signaling, which possibly is the underlying cause for the manifold downstream effects of Fgf signaling.

Further support for a possible redundancy of the Fgf receptors in the mid-/hindbrain region comes from previous reports on dosage dependent defects in an allelic series of *Fgf8* mutant mice. Hypomorphs, compound hypomorphic/null and complete null mutants for *Fgf8* revealed a graded sensitivity of mid-/hindbrain structures to reduced amounts of *Fgf8* transcripts (Meyers et al., 1998; Chi et al., 2003). The phenotype of *Fgf8* hypomorphic mutants is reminiscent of the defects observed in conditional *Fgfr1* KOs (Trokovic et al., 2003). *Fgf8* hypomorphic/null compounds in addition also loose the SC and the TH expressing cells of the ventral mid-/hindbrain region, while *Fgf8* conditional mid-/hindbrain region mutants loose the complete mid-/hindbrain region (Chi et al., 2003). Overlapping expression (Blak et al., 2005) and redundant function of *Fgfr1*, *Fgfr2* and *Fgfr3* in the ventral mid-/hindbrain region could keep Fgf signaling above a threshold that is necessary for proper development of ventral tissues in the *Fgf8* hypomorphic mutants but not in *Fgf8* hypomorphic/null compound and conditional Fgf8 mid-/hindbrain region mutants.

Besides alterations on the transcriptional level, posttranslational modifications could also contribute to compensation of the loss of *Fgfr2* or *Fgfr3* in the mid-/hindbrain region. It has recently been shown that with partially impaired Fgfr1 signaling, basal levels of active Fgfr2 are elevated (Hoch and Soriano, 2006). This may hint towards a transregulation of at least the Fgfr2 via Fgfr1 signaling on the posttranslational level in order to preserve homeostasis with respect to Fgfr signaling.

Similar transregulation might also exist for other combinations of the Fgf receptors. Loss of an Fgf receptor could also be compensated by a reduction in inhibitory feedback on Fgf signaling from factors downstream of the Fgf receptors, such as MKP3, Sprouty1 or Sef1. This would only be possible if a second Fgf receptor can replace the lost one and further transmit Fgf signals. While we could not find a down-regulation of MKP3, Sprouty1 or Sef1 on the transcriptional level, it would be interesting to study the translation or phosphorylation of these factors in the conditional *Fgfr2* and *Fgfr3* knockout mice.

Taken together, we showed that despite the fact that both receptors are expressed in close vicinity to the *Fgf8* expression domain in the anterior hindbrain, and that the conditional knockout of the *Fgfr1* in the mid-/hindbrain region did not exhibit a severe loss of tissue in the ventral mid-/hindbrain region, the conditional *Fgfr2* knockout and the *Fgfr3* knockout do not show an obvious phenotype in the mid-/hindbrain region. This implies that the Fgfrs 1, 2 and 3 act in a redundant manner, which has to be proven by the analysis of double and triple knockouts of these receptors in the mid-/hindbrain region.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2006.11.008.

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