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Specific and redundant functions of Fgf receptors in development of the midbrain and anterior hindbrain of the mouse

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Contents

1 Introduction

1.1 Description of the adult midbrain and hindbrain

The midbrain and hindbrain are important structures for the transduction, integration and modulation of information that is exchanged between the periphery (sensory organs) and the cortex and diencephalon. The dorsally located tectum of the midbrain consists of the superior and inferior colliculi (Fig. 1a). The superior colliculi (SC) integrate information from sensory organs (e.g. the eyes and ears), the cortex and from other sensory structures that belong to the visual system and coordinate the movement of the head and the eyes. The inferior colluculi (IC) form a relay station that preprocesses auditory sensory information and provides output to higher brain areas. In the dorsal hindbrain, the cerebellum (Fig. 1a) receives motor information from the cortex and sensory information from the periphery and serves as a feedback center that regulates voluntary movements. It is also important for the learning of stereotypic movements. The ventral midbrain-/hindbrain region (MHR) contains a group of neuronal nuclei that are important for the regulation of movements and for the modulation of higher brain functions, such as mood, reward and learning. The ventral tegmental area (VTA) and the substantia nigra (SN) in the ventral midbrain comprise the major populations of dopaminergic (DA) neurons in the brain (Fig. 1b). Dopaminergic neurons of the VTA are found in the midline of the ventral midbrain while those of the SN are positioned laterally on both sides of the VTA. The main projection targets of the VTA are the nucleus accumbens and the cerebral cortex (mesocorticolimbic pathway; Fig. 2A). Dopamine release from the mesocorticolimbic pathway regulates motivation and reward anticipation (Nestler, 2001; Hyman and Malenka, 2001; Salamone and Correa, 2002). Dopaminergic neurons of the SN innervate the striatum (nigrostriatal pathway; Fig. 2A) and regulate the activation of movements (Groenewegen, 2003). Loss of midbrain DA (mDA) neurons in the SN is one hallmark of Parkinson's Disease and is thought to be the cause of the motor symptoms observed in Parkinson's patients (Moore et al., 2005). A promising strategy for the treatment of Parkinson's Disease is the replacement of dopaminergic cells, either by transplantation of in vitro differentiated dopaminergic neurons or by the directed differentiation of endogenous neuronal progenitor cells (Fisher, 1997; Zoghbi et al., 2000). Attention deficit hyperactivity disorder, a syndrome that is common in children and adults, drug abuse and Schizophrenia have been linked to an imbalance in dopamine production (Kienast and Heinz, 2006; James et al., 2007).

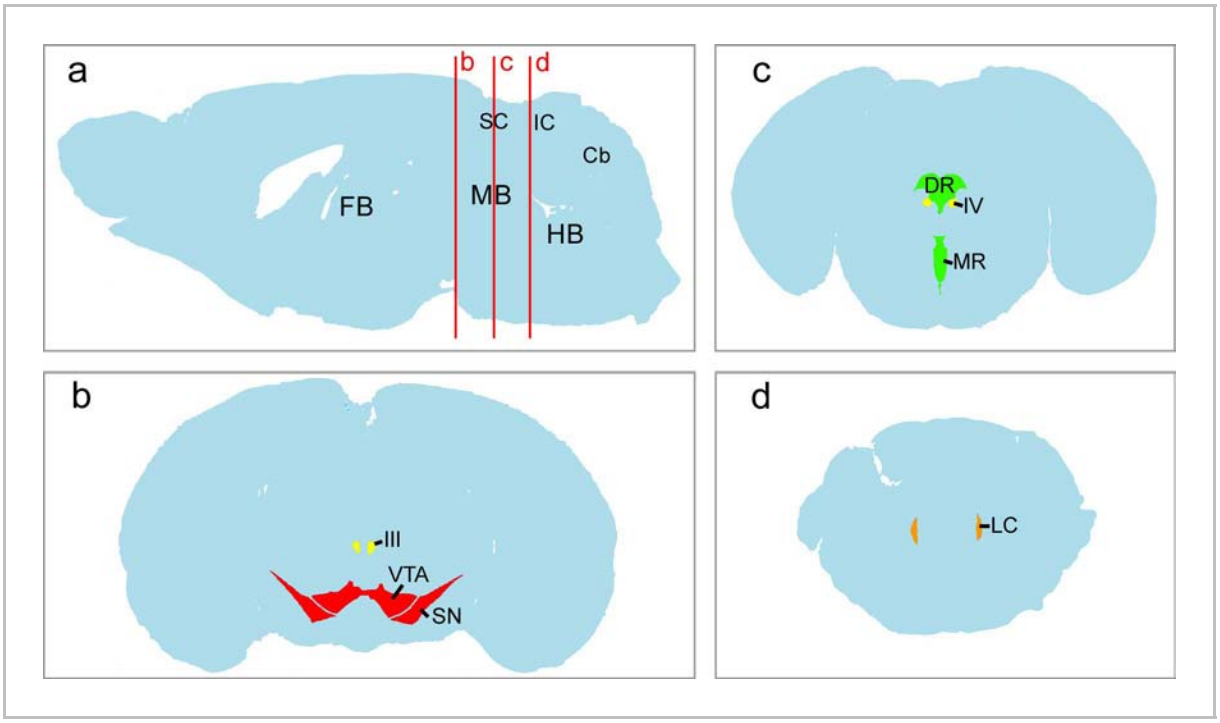

Fig. 1 Distribution of neuronal cell populations in the MHR of the adult mouse brain. a Schematic sagittal view of a mouse brain, showing the segmentation of the brain and the major structures of the dorsal MHR. In the dorsal midbrain (tectum) the SC and IC are prominent structures. The Cerebellum is the main component of the dorsal hindbrain. Red lines depict the levels of the corresponding coronal sections in **b**,**c** and **d**. FB: forebrain, MB: midbrain, HB: hindbrain, SC: superior colliculus, IC: inferior colliculus, Cb: Cerebellum. **b** In the ventral midbrain, DA neurons are found in the lateral SN and in the medial VTA (red). The cholinergic neurons of the 3rd cranial nerve reside in the midbrain, dorsal to the VTA (yellow). SN: substantia nigra, VTA: ventral tegmental area, III: nuclei of 3rd cranial nerve (oculomotorius). **c** The DR and MR contain 5-HT neurons (green) and are found in the midline of the anterior hindbrain. The nuclei of the 4th cranial nerve are located laterally to the DR and contain cholinergic neurons (yellow). DR: dorsal raphe nucleus, MR: medial raphe nucleus, IV: nuclei of 4th cranial nerve. **d** The noradrenergic neurons of the LC (orange) are positioned in the caudal pons. LC: locus coeruleus.

In the ventral hindbrain, the serotonergic (5-HT) neurons of the CNS are located in the raphe nuclei. One major group of 5-HT neurons, the dorsal raphe (DR) nucleus, is found in the adult ventral hindbrain (Fig. 1c). Further ventral, 5-HT neurons are located in the medial raphe (MR) nucleus of the anterior hindbrain (Fig. 1c). Serotonergic neurons of the DR and MR form diffuse projections to the cortex and to structures of the limbic system, such as the amygdala (Hensler, 2006) (Fig. 2B) and have been shown to be involved in the regulation of anxiety and awareness (Abrams et al., 2004). 5-HT neurons of the DR have been associated with depression, drug addiction and autism (Gaspar et al., 2003; Gingrich et al., 2003; Michelsen et al., 2007).

Thus, prevalent diseases have been linked to altered function of the dopaminergic and serotonergic systems in the mid-/hindbrain region.

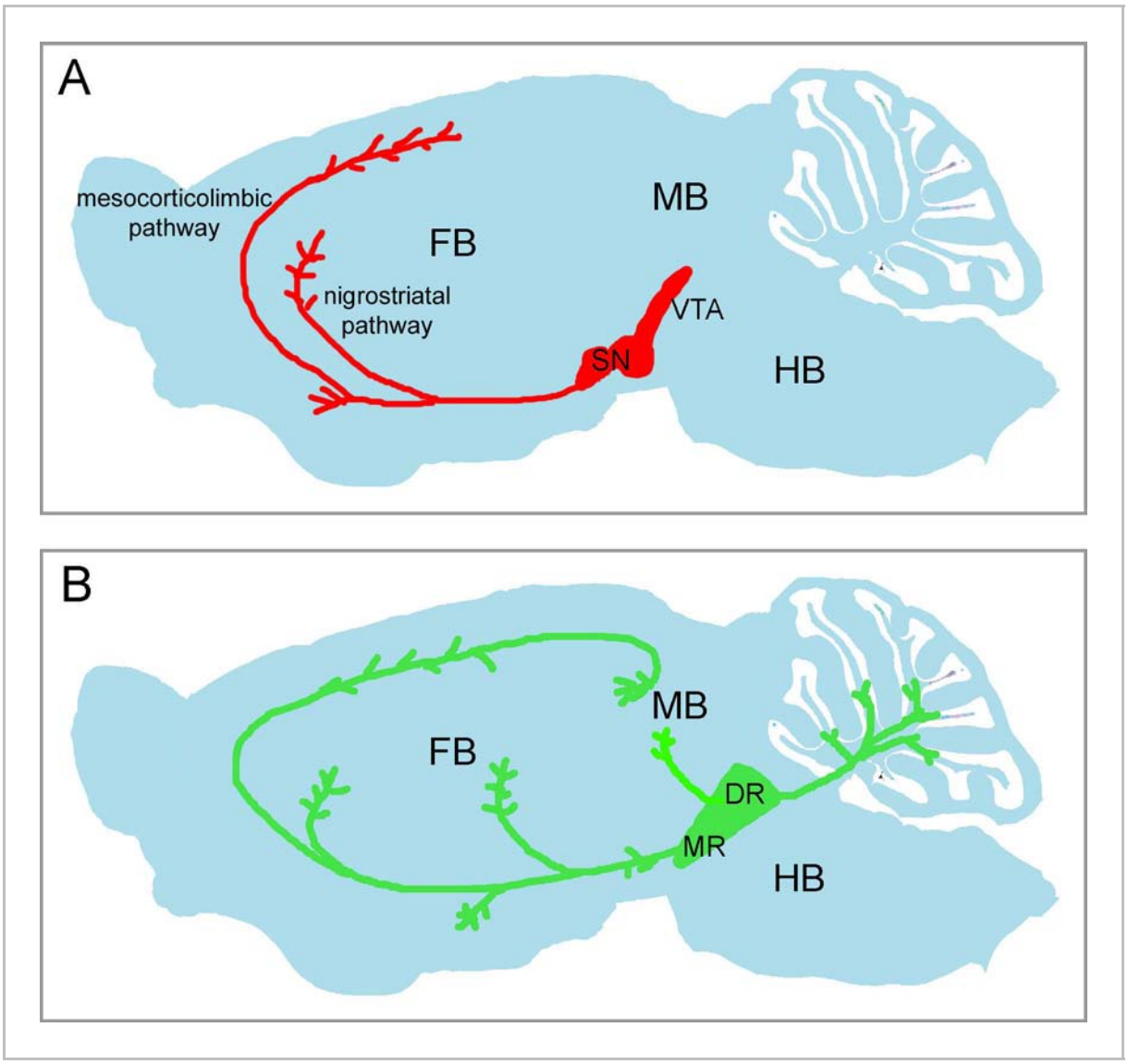

Fig. 2 Projections of dopaminergic and serotonergic neurons from the ventral MHR. A mDA neurons (red) of the SN project to the striatum (nigrostriatal pathway), whereas mDA neurons of the VTA project to the nucleus accumbens and to the cortex (mesocorticolimbic pathway). **B** 5-HT neurons of the DR and MR diffusely innervate many target areas in the brain. FB: forebrain, MB: midbrain, HB: hindbrain, SN: substantia nigra, VTA: ventral tegmental area, DR: dorsal raphe nucleus, MR: medial raphe nucleus.

Some of the nuclei that belong to the cranial nerves reside also in the ventral midbrain and hindbrain, among them the nucleus oculomotorius (nucleus of 3rd cranial nerve; Fig. 1b) and the nucleus trochlearis (nucleus of the 4th cranial nerve; Fig. 1c). The locus coeruleus (LC) is located in the ventral hindbrain (Fig. 1d) and provides diffuse noradrenergic projections

toward the cortex and other higher brain areas. It is involved in the regulation of arousal and attention (Aston-Jones et al., 2001).

1.2 Development of the MHR

To understand the etiology of diseases associated with changes in neuronal populations that develop in the MHR, it is of high importance to know how the MHR develops in the embryo. Knowledge about the mechanisms which regulate normal development of the MHR and its neuronal populations will also help in the search for new therapeutical strategies, e.g. for DA cell replacement in Parkinson's Disease patients.

1.2.1 The Midbrain-/Hindbrain Organizer and patterning of the MHR

Development of the midbrain and hindbrain is governed by a secondary organizing center within the neural tube, the so called mid-/hindbrain organizer (MHO), which is located at the isthmic constriction, the interface between the midbrain and the hindbrain. The MHO is characterized by the expression of a set of transcription factors and secreted molecules that are important for its function (Rhinn and Brand, 2001; Wurst and Bally-Cuif, 2001; Liu and Joyner, 2001a; Echevarria et al., 2003). Interaction of the two transcription factors Orthodenticle like homeobox protein 2 (Otx2) and Gastrulation brain homeobox protein 2 (Gbx2) determines the position of the MHO along the anterior/posterior axis (a/p) around embryonic day (E)8 (Broccoli et al., 1999; Millet et al., 1999; Simeone, 2000). Subsequently, the expression of the transcription factors *Engrailed 1* and *2* (*En1/2*) and *Paired domain homeobox proteins 2* and *5* (*Pax2/5*) is induced across the midbrain-/hindbrain boundary (Rowitch and McMahon, 1995). En1, En2, Pax2 and Pax5 have all been shown to be necessary for the normal development of the MHR (Wurst et al., 1994; Millen et al., 1994; Urbanek et al., 1997; Simon et al., 2001). Among the secreted factors that control development of the MHR are the growth factors Wingless-type MMTV integration site 1 (Wnt1), Sonic hedgehog (Shh) and Fibroblast growth factor 8 (Fgf8), which cooperate in the establishment and maintenance of a regional code that regulates positioning and fate of neuronal cell populations in the MHR. *Wnt1* is expressed as a ring in the caudal midbrain, immediately anterior to the mid-/hindbrain boundary, and also along the midline of the dorsal and in two parallel stripes in the ventral midbrain and has been shown to be necessary for development of tissues and neuronal cell populations in the MHR (McMahon et al., 1992;

Prakash et al., 2006). *Shh* is expressed along the floor plate of the neural tube and in the midbrain its expression extends into the basal plate (Prakash and Wurst, 2004). Several neuronal populations in the ventral MHR have been suggested to depend on the expression of *Shh* (Hynes et al., 1995; Ye et al., 1998; Hynes and Rosenthal, 2000; Lam et al., 2003). *Fgf8* is expressed as an open ring in the most anterior r1, directly posterior to the mid-/hindbrain boundary, excluding the floor plate. Morphogenesis of the MHR requires *Fgf8* expression (Meyers et al., 1998; Chi et al., 2003). Furthermore, the ectopic application of FGF8 protein is sufficient to induce the expression of MHO genes and the formation of ectopic hindbrain and midbrain structures in the diencephalon and posterior hindbrain (Crossley et al., 1996; Liu et al., 1999; Martinez et al., 1999). Besides *Fgf8*, also other Fgfs are expressed in the developing MHR. These include *Fgf15*, *Fgf17* and *Fgf18*. Fgf15 has a function in the regulation of proliferation and cell cycle progression and is not restricted to the MHR (Gimeno et al., 2002; Fischer, 2006). For Fgf17, an involvement in the development of the anterior cerebellum has been shown (Xu et al., 2000). Fgf18 might contribute to the regulation of proliferation in the MHR, but loss of *Fgf18* in the MHR does not lead to any obvious defects (Ohbayashi et al., 2002; Liu et al., 2003).

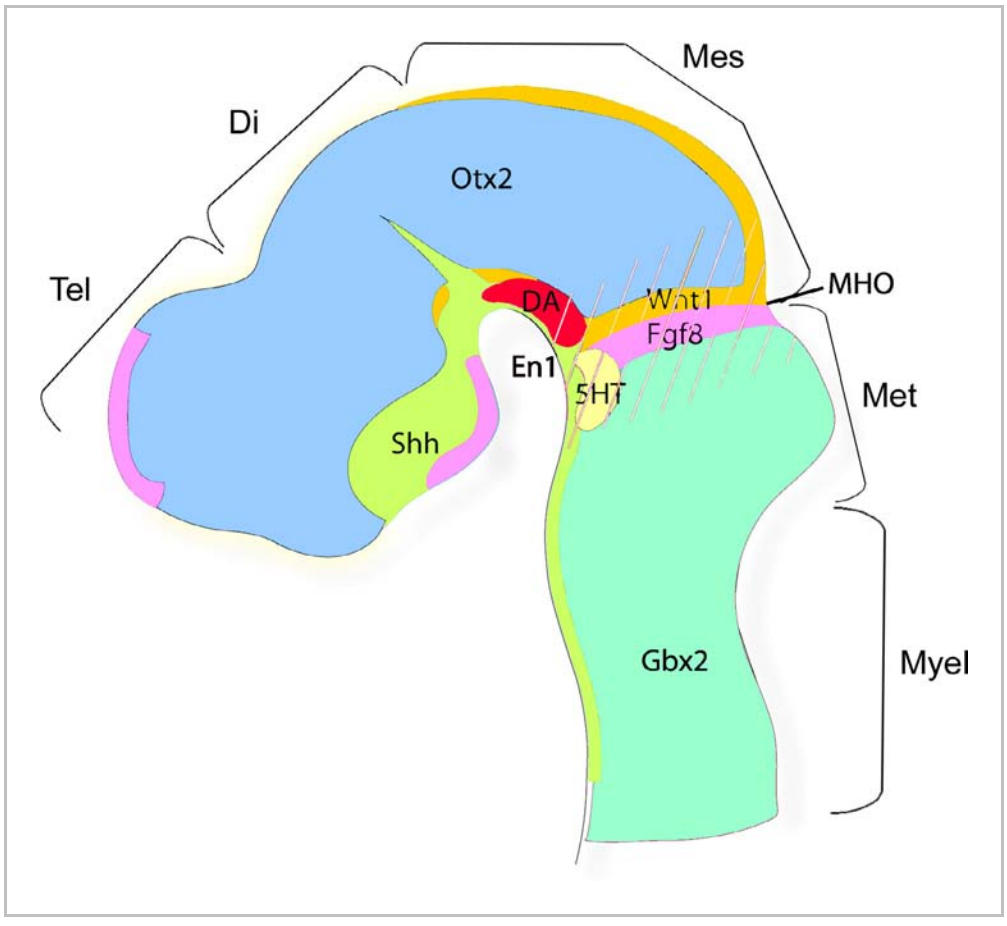

Fig. 3

Fig. 3 Schematic sagittal view of the E11 embryonic mouse brain. The subdivision of the anterior neural tube into the brain regions (Tel-, Di-, Mes-, Met- and Myelencephalon) is apparent at midgestational stages in mouse embryos. Expression of *Otx2* (blue) demarcates the anterior brain (Tel-Mes), whereas *Gbx2* expression (turquoise) is prominent in the caudal brain (Met, Myel). The interface of the *Otx2* and *Gbx2* expression domains positions the MHO. A/p patterning in the MHR is dependent on *Wnt1* expression (orange) in the midbrain and on *Fgf8* expression (purple) in the hindbrain. *Shh* (light green) serves to pattern the neural tube along the d/v axis. *En1* (white stripes) is expressed across the MHO and is involved in maintenance of the MHR. In the ventral midbrain the precursor cells of mDA neurons (red) are located just anterior to the MHO. 5-HT precursor cells (green) arise immediately posterior to the MHO. Tel: Telencephalon, Di: Diencephalon, Mes: Mesencephalon, Met: Metencephalon, Myel: Myelencephalon, DA: dopaminergic precursor cells, 5-HT: serotonergic precursor cells; MHO: mid-/hindbrain organizer.

1.2.2 Development of neuronal populations in the MHR

1.2.2.1 Timing of neuronal differentiation in the ventral MHR

Neuronal populations in the ventral MHR are generated at slightly different time points during embryonic development. The first noradrenergic neurons can be detected at E9.5 in the LC of the anterior hindbrain (Steindler and Trosko, 1989; Porzio et al., 1990; Aroca et al., 2006). The next neuronal populations differentiating in the ventral MHR are the cholinergic motoneurons of the 3rd and 4th cranial nerves, which begin to differentiate between E12 and E13 in rat embryos (Altman and Bayer, 1981), corresponding to E10.5 - E11.5 in the mouse (Tsuchida et al., 1994) (http://embryology.med.unsw.edu.au/OtherEmb/CStages.htm). Starting at E11, differentiating dopaminergic precursor cells are found in the ventral midbrain. Serotonergic neurons in the anterior ventral hindbrain can be detected from E11.5 onwards (Goridis and Rohrer, 2002).

1.2.2.2 Development of dopaminergic neurons in the MHR

Recent publications greatly enhanced our knowledge of the growth factors and transcriptional regulators that account for the specification and differentiation of midbrain dopaminergic neurons (Prakash and Wurst, 2006; Ang, 2006; Andersson et al., 2006a; Andersson et al., 2006b). The earliest marker known for progenitors of midbrain dopaminergic neurons is the *Retinaldehyde dehydrogenase 1* (*Raldh1/Aldh1a1*) gene. *Raldh1* is exclusively expressed in dopaminergic neurons in the adult ventral midbrain and starts to be expressed in the embryonic ventral midbrain at E9.5, suggesting that midbrain

dopaminergic progenitor cells become specified about this time point (Wallen et al., 1999; Prakash and Wurst, 2006). The first differentiating precursors of midbrain dopaminergic neurons can be visualized by in-situ hybridization for *Tyrosine hydroxylase* (*TH*) around E10.5 in mouse embryos (Marin et al., 2005), but TH protein can only be detected after E11. Fully mature mDA neurons are characterized by the expression of the *Dopamine transporter* (*Dat/Slc6a3*), which is first detectable at E12.5 (Brodski et al., 2003) and indicates the appearance of terminally differentiated dopaminergic neurons in the ventral midbrain. Several genes expressed in the midbrain and at the mid-/hindbrain organizer have been suggested to participate in the specification and differentiation of midbrain dopaminergic neurons. Experiments on explants from rat brain suggested an involvement of FGF8 and SHH in the specification of dopaminergic neurons (Ye et al., 1998). Recently, we could show that Wnt1 is involved in the specification of midbrain dopaminergic neurons and that it is also necessary for the terminal differentiation of these cells (Prakash et al., 2006). Transcription factors acting downstream of Fgf8, Wnt1 and Shh include the LIM homeobox transcription factors 1 alpha (Lmx1a) and beta (Lmx1b). Expression of *Lmx1a* can first be detected in the ventral midline of the midbrain at E9. Lmx1a is the only transcription factor that has been suggested to have an instructive role in the specification of midbrain dopaminergic neurons (Andersson et al., 2006b). *Lmx1b*, which starts to be expressed in the ventral MHR before E8 (Burbach et al., 2003), is probably indirectly involved in the generation and maintenance of dopaminergic neurons by transcriptional upregulation of *Fgf8* and *Wnt1* (Adams et al., 2000; Guo et al., 2006). The orphan *Nuclear receptor related protein 1* (*Nurr1/Nr4a2*) starts to be expressed in dopaminergic precursor cells at E10.5, the time when *TH* expression is also induced in the ventral midbrain (Perlmann and Wallen-Mackenzie, 2004). Indeed, *Nurr1* is important for the induction of *TH* and *Dat* expression during the maturation of midbrain dopaminergic precursors (Zetterstrom et al., 1997; Saucedo-Cardenas et al., 1998; Wallen et al., 1999; Smits et al., 2003). *Paired like homeodomain transcription factor 3* (*Pitx3*) is found exclusively in differentiating dopaminergic neurons in the ventral midbrain from E11 on and is required for the expression of *TH* and for the differentiation and survival of mDA neurons (Lebel et al., 2001; van den Munckhof et al., 2003; Smidt et al., 2004; Maxwell et al., 2005; Smits et al., 2005).

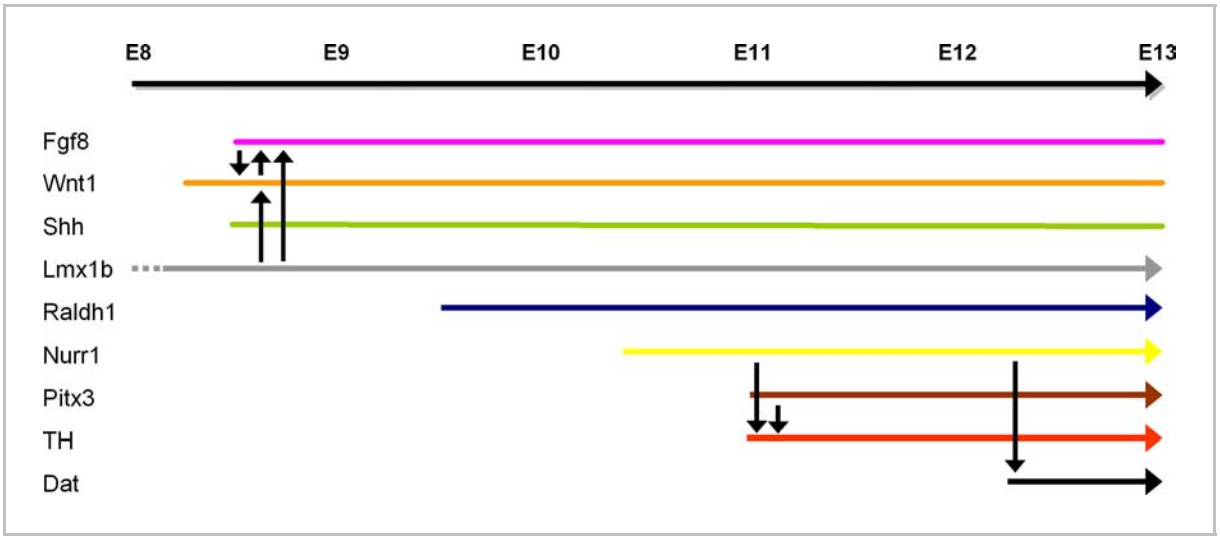

Fig. 4 Timescale showing the expression of factors related to development of midbrain dopaminergic neurons. Successive expression of the patterning factors *Wnt1*, *Fgf8* and *Shh* is necessary for the induction of transcription factors that are expressed in precursor cells of mDA neurons. *Lmx1b* is the earliest transcription factor expressed in mDA progenitors. Expression of *Raldh1*, *Nurr1* and *Pitx3* follows that of *Lmx1b* at later time points. Differentiation of mDA neurons begins around E11 with the production of TH protein and final maturation takes place at about E12.5, when *Dat* expression is first detectable in the ventral MHR. Black arrows indicate positive regulation of genes.

1.2.2.3 Development of serotonergic neurons in the MHR

During the last 10 years, the molecular mechanisms that drive the specification and differentiation of 5-HT neurons in the hindbrain have been studied extensively (Cordes, 2005). Therefore, the major factors and the sequence of events that lead to the formation of serotonergic neurons in r2,3 and r5-7 of the hindbrain have become clear (Fig. 5). But it also became obvious that the anterior serotonergic neurons in r1 develop differently from the 5-HT neurons in the caudal rhombomeres (van Doorninck et al., 1999; Briscoe et al., 1999; Pattyn et al., 2003; Craven et al., 2004; Cordes, 2005). Caudal 5-HT precursors depend on the induction of the transcription factor *Nkx2.2* by Shh (Briscoe et al., 1999). Nkx2.2 in turn is required for the expression of *Gata-2* and *Gata-3*, which participate in the induction of genes that make up the mature phenotype of 5-HT neurons (van Doorninck et al., 1999; Craven et al., 2004). Anterior 5-HT neurons can be generated by the combined action of Shh, Fgf8 and Fgf4 and a dependence of serotonergic neurons on Hh and Fgf signaling has been reported from experiments in zebrafish (Ye et al., 1998; Teraoka et al., 2004). The differentiation of all 5-HT neurons depends on the expression of a common set of transcription factors. Lmx1b is required for the maintenance of 5-HT neurons, probably because it is necessary for the

maintenance of *Pet1* expression (Ding et al., 2003; Zhao et al., 2006). The earliest differentiating 5-HT neurons in the hindbrain specifically express *Pet1*, a transcription factor of the ETS-family (Hendricks et al., 1999; Pfaar et al., 2002). In *Pet1* knock out mice the majority of 5-HT neurons in the hindbrain is absent and the remaining cells do not express all markers that are characteristic for 5-HT neurons, suggesting an involvement of Pet1 in the terminal differentiation of serotonergic neurons (Hendricks et al., 2003). Mouse achaete scute homolog 1 (Mash1) has been shown to be required for the expression of *Pet1* and the *Gata transcription factors* in differentiating serotonergic precursor cells (Pattyn et al., 2004). Terminally differentiated 5-HT neurons are defined by their ability to produce Serotonin (5-HT) and by the expression of several genes involved in the synthesis, reception and reuptake of 5-HT (Goridis and Rohrer, 2002). An example for a characteristic marker of mature 5-HT neurons is the *Serotonin reuptake transporter* (Sert) (Pfaar et al., 2002). The focus in this dissertation was on the development of anterior 5-HT neurons in r1.

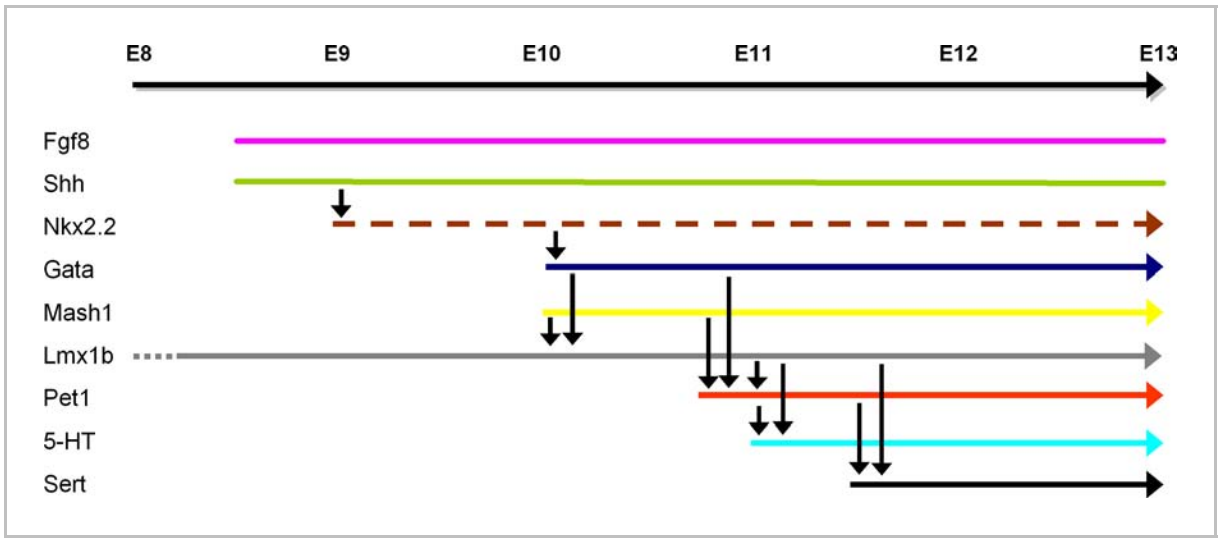

Fig. 5 Timescale showing the expression of factors related to development of hindbrain serotonergic neurons. *Fgf8* and *Shh* are the first growth factors expressed in the neuroepithelium that have been suggested to be involved in the specification of 5-HT neurons. The earliest transcription factor implicated in 5-HT development is Lmx1b. Induction of *Gata transcription factors* and *Mash1* follows that of *Nkx2.2*. The dashed line indicates that Nkx2.2 is only necessary for the development of caudal 5-HT neurons in r2,r3 and r5-7. Expression of *Pet1* is first detectable at E10.75 and shortly precedes the synthesis of 5-HT, which is synthesized from E11 onwards. After E11.5, *Sert* is expressed in terminally differentiated 5-HT neurons. Black arrows indicate positive regulation of genes.

1.3 The role of Fgfs and Fgf receptors in the MHR

1.3.1 Fgfs have important functions in patterning, specification and maintenance in the midbrain-/hindbrain region.

The mid-/hindbrain organizer consists of a complex regulatory network of genes expressed at the mid-/hindbrain boundary. Loss of any of these molecules, including *Fgf8*, leads to an early loss of the mid-/hindbrain organizer and consequently to a loss of the MHR (McMahon et al., 1992; Urbanek et al., 1997; Simon et al., 2001; Chi et al., 2003). In-vitro studies using recombinant FGF8 protein on brain explants have demonstrated that FGF8 is able to induce the expression of *Gbx2* and concomitantly the repression of *Otx2*. Application of FGF8 protein also leads to induction of *Wnt1* expression (Liu et al., 1999). In conditional mutants for *Fgf8*, which loose *Fgf8* expression in the midbrain-/hindbrain region around E9, the expression of mid-/hindbrain genes (*Gbx2*, *Pax2*, *En1*, *Fgf17/18*) was lost and led to increased cell death and a complete loss of the MHR (Chi et al., 2003). Besides its role in the maintenance of the mid-/hindbrain organizer, Fgf8 is the only factor that is known to mediate the patterning function of the midbrain-/hindbrain organizer. Ectopic application of FGF8 to the diencephalon or to the hindbrain leads to induction of genes expressed at the isthmus and to the ectopic formation of midbrain and hindbrain structures (Martinez et al., 1999; Shamim et al., 1999). Similar induction of mid-/hindbrain organizer genes and of midbrain and hindbrain structures have been observed after electroporation of an *Fgf8* expression construct into the MHR (Liu et al., 2003; Sato and Nakamura, 2004). Fgf8 is also involved in the specification of neuronal populations in the MHR. In rat diencephalon explants that were treated with FGF8 coated beads, markers for midbrain dopaminergic neurons were induced in the presence of Shh (Ye et al., 1998). Fgf4 is an Fgf that is expressed in the primitive streak mesoderm, underlying the hindbrain neuroectoderm. In metencephalic rat brain explants, Fgf4 was found to be necessary for the induction of serotonergic neurons (Ye et al., 1998). Two other Fgfs that belong to the same group as Fgf8 are Fgf17 and Fgf18 (Maruoka et al., 1998; Xu et al., 1999). Both are expressed across the midbrain-/hindbrain boundary during midgestational development. While knock out mutants for *Fgf18* did not show defects in the MHR (Ohbayashi et al., 2002), knock out mice for *Fgf17* showed a reduction in size of the inferior colliculi and of the anterior cerebellar vermis. On an *Fgf8* heterozygous knock out background, the loss of *Fgf17* led to more severe defects in anterior cerebellar foliation and to an increased reduction in size of the IC, demonstrating a partial redundancy of these two Fgfs in dorsal mid-/hindbrain development (Xu et al., 2000). Thus, Fgfs are important factors

for the development of the MHR, with Fgf8 being essential for the function of the midbrain-/hindbrain organizer.

1.3.2 The role of Fgf receptors in mid-/hindbrain development is not fully understood

While Fgf8 has been shown to be essential for the correct formation and maintenance of the MHR (Chi et al., 2003), not much is known about the role of Fgf receptors in MHR development. Fgf receptors belong to the family of tyrosine kinase transmembrane receptors. Four transmembrane Fgf receptors (Fgfr1-4) are known that bind Fgfs in combination with heparane sulfate proteoglycans and transmit signals into the receiving cell (Reuss and Bohlen und, 2003). Expression of *Fgf receptors* in the MHR of the mouse has been described before (Ishibashi and McMahon, 2002; Trokovic et al., 2003; Liu et al., 2003). According to these reports, *Fgfr1* is expressed throughout the embryonic MHR, but *Fgfr2* and *Fgfr3* have not been detected in the posterior midbrain or anterior hindbrain. Due to these descriptions of Fgfr expression in the MHR, Fgfr1 was believed to mediate the function of the Fgfs in the MHR during development. Analyses on the function of Fgfr1 in the MHR were performed on conditional $En1^{Cre/+}$ $Fgfr1^{l/l}$ mouse mutants, in which the *Fgfr1* gene was inactivated in the MHR after E9 (Trokovic et al., 2003). Surprisingly, the $En1^{Cre/+}$ $Fgfr1^{l/l}$ mice were viable and fertile, although they showed an abnormal gait and had problems with balance. Furthermore, they exhibited a loss of the IC and of the cerebellar vermis, but structures derived from further anterior or posterior tissue of the dorsal MHR (i.e., the superior collliculi and the hemispheres of the cerebellum) were intact and no loss of ventral tissue was observed (Trokovic et al., 2003). The only defect detected in the ventral MHR of conditional *Fgfr1* mutants was a disorganization of the locus coeruleus, where neurons were slightly dispersed and not as compact as in wildtype (wt) animals (Trokovic et al., 2003). At early developmental stages, a downregulation of Fgf8 dependent genes, including *En1* and *Pax2,* was described in the MHR of the $En1^{Cre/+}$ $Fgfr1^{l/l}$ mutant mice, but other genes expressed at the mid-/hindbrain boundary, such as *Otx2*, *Wnt1* and *En2*, were not affected (Trokovic et al., 2003). This is in contrast to the conditional knock out of *Fgf8* in the MHR, which was generated using the same $En1^{Cre/+}$ line. *Fgf8* MHR conditional knock out mice lose the complete MHR at early stages of development, due to loss of MHO gene expression and subsequent cell death in the region. The tissue loss includes the dorsal SC and cerebellar hemispheres as well as the ventral MHR, containing the VTA and SN, the LC and the 3rd and 4th cranial nerve (Chi et al., 2003). The discrepancy between the *Fgfr1* and *Fgf8* conditional MHR knock out mice suggests that Fgf signaling in the developing MHR is not exclusively mediated by Fgfr1. Other Fgf receptors might be expressed in the region, especially in the

tissues that are not affected in the *Fgfr1* conditional knock out. These receptors could take over transduction of Fgf signals to rescue the development of the tissues that are not affected in the conditional *Fgfr1* knock outs. A prerequisite for this hypothesis is a functional redundancy of the Fgf receptors in the MHR. However, disorganization of the LC in the ventral MHR of *Fgfr1* conditional knock outs also hinted to specific roles for Fgfr1 alone, in the development of ventral mid-/hindbrain structures.

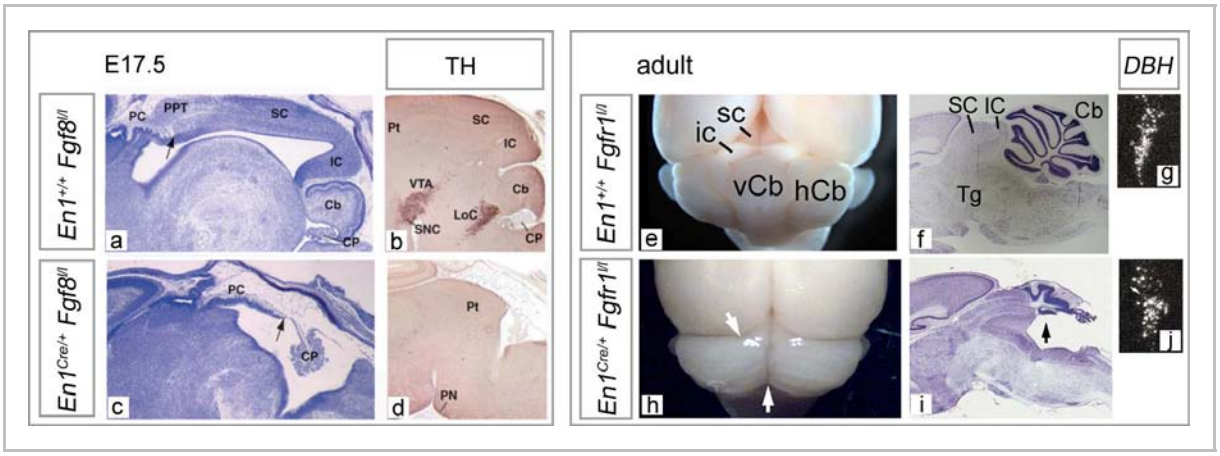

Fig. 6 Defects in *En1*$^{Cre/+}$ *Fgf8*$^{l/l}$ mutants are more severe than those in *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ mice. **a** The dorsal MHR consists of the pPT, the SC, the IC and the Cb. a In wildtype (*wt*) embryos at E17.5 these structures are clearly distinguishable. **b** Immunohistochemistry reveals staining for TH in the ventral midbrain (VTA and SN) and hindbrain (LC) of *wt* embryos at E17.5. **c** *En1*$^{Cre/+}$ *Fgf8*$^{l/l}$ mutants lose all structures in the dorsal MHR. **d** Ventral nuclei are also absent from the MHR of *En1*$^{Cre/+}$ *Fgf8*$^{l/l}$ embryos, as no TH staining is detectable in that region of the mutants. Arrows mark caudal end of posterior commissure. **e** Top view of an *wt* adult mouse brain, showing the SC, IC, the vermis and hemispheres of the Cb. **f** Sagittal section through an *wt* adult mouse brain. The SC, IC, cerebellar vermis and the tegmentum are labelled. **g** In-situ hybridization for *DBH* shows the noradrenergic neurons of the wt LC. **h** *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ mutant mice lose the IC and the vermis of the Cb in the dorsal MHR. **i** In a sagittal section of an *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ mutant brain, the IC and vermis of the Cb are clearly absent (only part of a hemisphere is visible). **j** However, ventral tissue is not lost in the MHR of an *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ mouse, as *DBH* can still be detected in the mutants LC. Arrows indicate tissue loss. PC: posterior commisure, pPT: posterior pretectal nucleus, SC: superior colliculus, IC: inferior colliculus, Cb: cerebellum, CP: choroids plexus, SNC: substantia nigra pars compacta, LoC: locus coeruleus, Pt: pretectum, PN: pontine nucleus, vCb: vermis of cerebellum, hCb: hemisphere of cerebellum, Tg: tegmentum. Adapted from Chi et al., 2003 and Trokovic et al., 2003.

1.4 Aim of the presented work

The aim of this thesis was to map the expression of candidate Fgf receptors that could substitute for Fgfr1 in the MHR and to test for functional redundancy and gene dosage dependency in an allelic series of conditional knock out mutants for *Fgfr1* and *Fgfr2*. Furthermore, a detailed analysis of the MHR was performed in $En1^{Cre/+}$ $Fgfr1^{l/l}$ mutants, with emphasis on the dopaminergic and serotonergic neurons of the ventral MHR, to reveal specific roles of Fgfr1 in ventral MHR development.

2 Results

2.1 Overview of the project

Ventral tissues were still present in *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ mutants which lack *Fgfr1* in the MHR, while *En1*$^{Cre/+}$ *Fgf8*$^{l/l}$ mice lost all of the MHR, including ventral tissues. The presence of ventral tissue in the MHR of the *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ mice allowed me to address the question, whether Fgf signaling by Fgfr1 has a specific role in the development of ventral neuronal populations *in vivo*. However, the discrepancies between the *Fgf8* and *Fgfr1* conditional knock out mice also led to the questions if there are other *Fgf receptors* expressed in the ventral MHR and whether functional redundancy might exist between Fgfr1 and other Fgf receptors in MHR development. To address these questions, three projects were pursued. First, I performed a detailed analysis of ventral patterning and cell populations in the *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ mutants. Then the expression of *Fgf receptors 1-3* in the developing MHR was mapped by radioactive *in-situ* hybridization in wildtype embryos at different developmental stages. And in the third project an allelic series of mutants for *Fgfr1* and/or *Fgfr2* in the MHR was generated to assess the possible degree of functional overlap of these two Fgf receptors in mid-/hindbrain development.

2.2 Analysis of the ventral mid-/hindbrain region in the *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ mouse

In the *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ mice, the only phenotypic change in the ventral MHR that had been described was a reduced density in the packing of LC neurons (Trokovic et al., 2003). Therefore, I investigated in detail the patterning and cell populations in the ventral MHR of *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ mutant brains. The purpose of this analysis was to find out whether Fgfr1 has specific roles during ventral MHR development.

2.2.1 Inactivation of *Fgfr1* in *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ mice

Mice with a conventional knock out of *Fgfr1* die because of gastrulation defects in early embryonic development (Yamaguchi et al., 1994; Deng et al., 1994). Therefore, I used a conditional knock out line of the *Fgf receptor 1* to investigate the role of Fgfr1 in the MHR.

Fgfr1$^{l/l}$ mice carrying two floxed alleles of *Fgfr1* (Trokovic et al., 2003) were crossed to heterozygous *En1*$^{Cre/+}$ mice (Kimmel et al., 2000), to inactivate *Fgfr1* in the MHR after E8.5. To validate the loss of the wildtype *Fgfr1* transcript in the mutants, a radioactive *in-situ* hybridization on sagittal sections from E10.5 embryos was performed. No wildtype transcript for *Fgfr1* was detected in the posterior midbrain and rhombomere 1 of the *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ embryos (Fig. 7a,b). Therefore, *Fgfr1* was assumed to be functionally inactivated. Similar results have been described by Trokovic et al. in 2003.

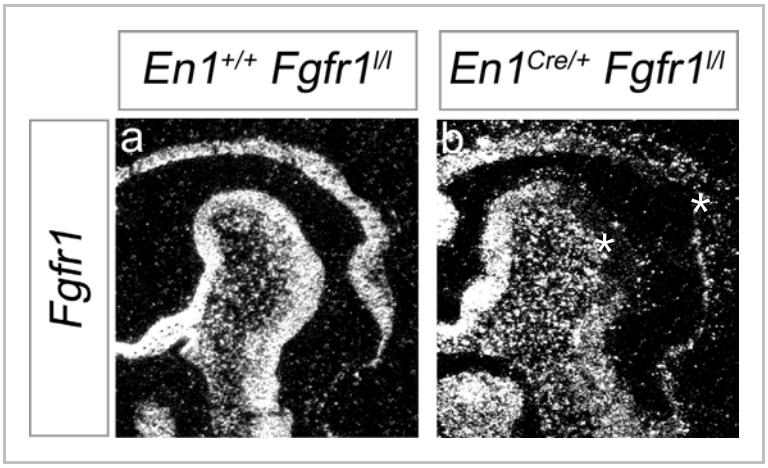

Fig. 7 *Fgfr1* is inactivated in the mid-/hindbrain region of *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ embryos at E10.5. Darkfield images of a wildtype (a) and an *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ (b) embryo, hybridized with probe for *Fgfr1*. *Fgfr1* is expressed throughout the neural tube of the control embryo (a), whereas no *Fgfr1* expression is detectable in the MHR of the mutant embryo (b). Asterisks mark the regions from which *Fgfr1* is absent in the mutant.

2.2.2 The expression of patterning genes is changed in the ventral MHR of *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ embryos

Correct patterning of the MHR is mediated by the growth factors *Fgf8*, *Wnt1* and *Shh*, but also by homeobox transcription factors, such as *Otx2* and *Gbx2*. Changes in the expression of one of these factors can also lead to altered specification of cell populations in the MHR. I performed radioactive *in-situ* hybridization experiments on sections from *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ and control embryos at the developmental stages E10.5 and E13.5, to compare the expression of genes involved in patterning of the MHR.

2.2.2.1 Expression of patterning genes intermingles at the ventral mid-/hindbrain boundary of *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ mutants at E10.5

E10.5 embryos were analyzed for the expression of patterning genes in the ventral MHR to check whether inactivation of *Fgfr1* leads to changes in patterning in this region. In control embryos, *Otx2* and *Wnt1* were expressed in the mesencephalic flexure with a sharp caudal expression boundary at the isthmus (Fig. 8a,b,c,d). *Otx2* and *Wnt1* were both expressed in the midbrain of *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ mutant embryos, but showed a speckled caudal boundary towards the hindbrain with patches of positive cells in the most anterior hindbrain (Fig. 8g,h,i,j). *Fgf8* expression in control animals was detected in the anterior r1 with a sharp anterior boundary at the isthmus, abutting the expression of *Otx2* and *Wnt1* (Fig. 8e,f). Complementary to the speckled expression of *Otx2* and *Wnt1* in the anterior hindbrain, patches of cells expressing *Fgf8* were found in the most caudal midbrain of mutant embryos (Fig. 8k,l). The mixture of cells with midbrain or hindbrain character around the mid-/hindbrain boundary hints towards a defect in sorting and/or migration of cells, as has been suggested in the first analysis of the *Fgfr1* conditional knock outs (Trokovic et al., 2003).

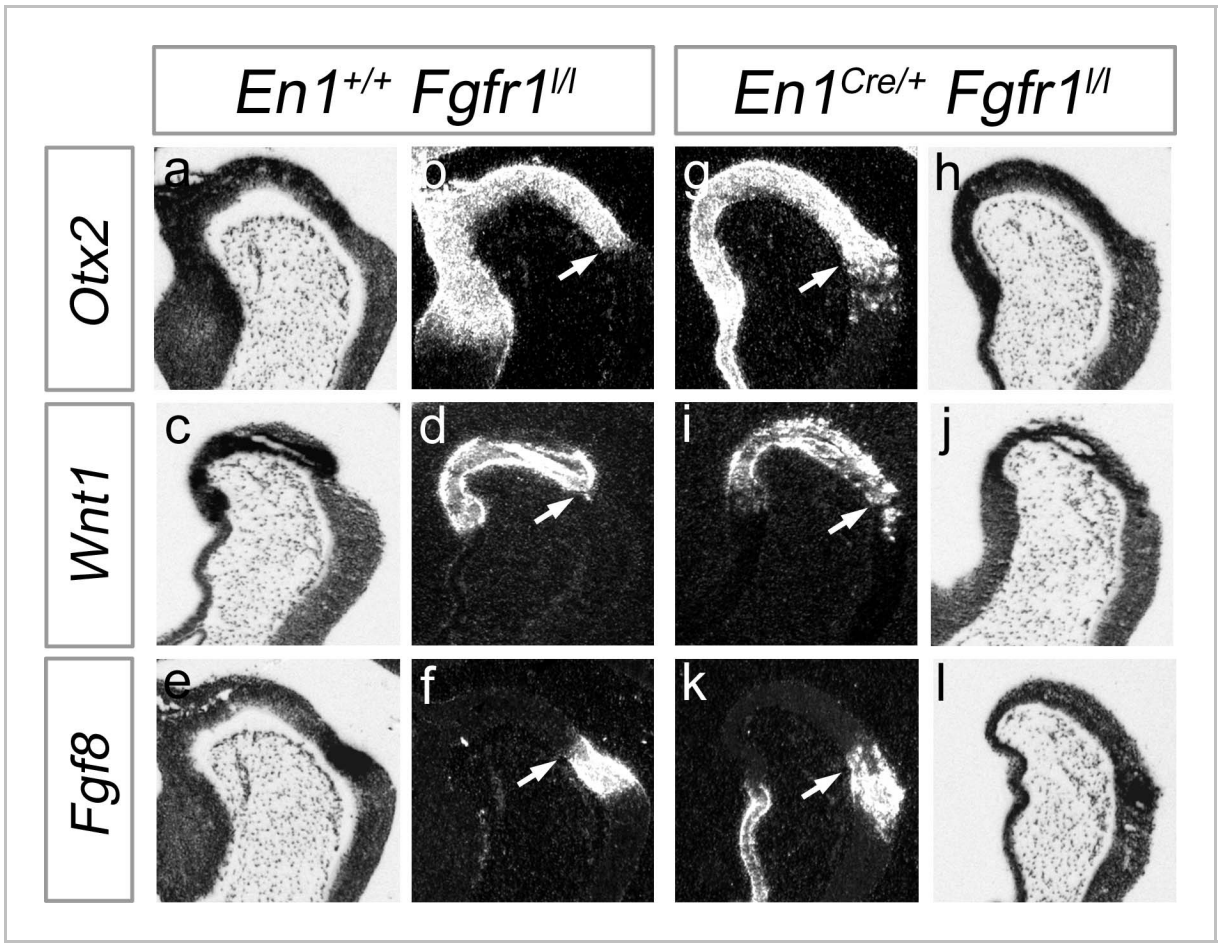

Fig. 8

Fig. 8 Expression of patterning genes at the ventral mid-/hindbrain boundary of *En1$^{Cre/+}$ Fgfr1$^{l/l}$* embryos intermingles at E10.5. Sagittal sections of the ventral mesencephalic flexure of wt (a-f) and mutant (g-l) embryos are shown. Patches of *Otx2* (b,g) and *Wnt1* (d,i) expression were found in the anterior hindbrain, while *Fgf8* (f,k) expression extended into the midbrain. The corresponding brightfield pictures to b,d,f and g,i,k are given in a,c,e and h,j,l respectively. Arrows demarcate the position corresponding to the wt midbrain-/hindbrain boundary.

2.2.2.2 Anterior patterning genes are expanded caudally in the ventral MHR of *En1$^{Cre/+}$ Fgfr1$^{l/l}$* mutants at E13.5

Failure in cell sorting at the mid-/hindbrain boundary in *En1$^{Cre/+}$ Fgfr1$^{l/l}$* embryos at E10.5 might have led to persistent alterations in patterning of the ventral MHR. Therefore, I analyzed the expression of patterning genes in the MHR at E13.5, a stage when neuronal populations in this region show robust differentiation and patterning genes are still expressed.

Empty spiracles homolog homeobox gene 2 (*Emx2*) is involved in patterning of the anterior neural tube. It is expressed in the ventral midbrain of wildtype embryos at E13.5 (Fig. 9b). In the mutants, expression of *Emx2* showed a diffuse caudal expansion into the anterior hindbrain (Fig. 9e). In wildtype mice, *Gastrulation brain homeobox gene 2* (*Gbx2*) is necessary for development of the hindbrain (Wassarman et al., 1997) and is expressed in two stripes along the caudal neural tube at E13.5. These stripes of *Gbx2* expression begin in r2 (Fig. 9d). Ventral expression of *Gbx2* in r2 was normal in the *En1$^{Cre/+}$ Fgfr1$^{l/l}$* mutants at E13.5 (Fig. 9g), suggesting that r2 development is not affected by the loss of *Fgfr1* in r1.

Expression of *Otx2* was still restricted to the anterior neural tube in wildtype embryos at E13.5, with a sharp caudal expression boundary at the isthmic organizer (Fig. 10a). In *En1$^{Cre/+}$ Fgfr1$^{l/l}$* embryos, patches of *Otx2* expression extended into the anterior ventral hindbrain at this stage (Fig. 10c), similar to the patches of ectopic *Otx2* expression that were found at E10.5. *NK2 transcription factor related 2* (*Nkx2.2*) is a homeobox gene that is found at the border between the basal and alar plate in the midbrain and in the most ventral basal plate in the hindbrain (Shimamura et al., 1995; Hartigan and Rubenstein, 1996). It has been implicated in the specification of serotonergic neurons (Puelles et al., 2004; Vernay et al., 2005; Prakash et al., 2006). As the expression of *Nkx2.2* in the hindbrain is located further ventral than in the midbrain, it shows a sharp anterior border at the mid-/hindbrain boundary of medial sections in wildtype embryos, abutting the *Otx2* expression domain (Fig. 10a,b). In *En1$^{Cre/+}$ Fgfr1$^{l/l}$* mutants, *Nkx2.2* expression in the hindbrain was shifted caudally, complementary to the caudal expansion observed in *Otx2* expression (Fig. 10c,d).

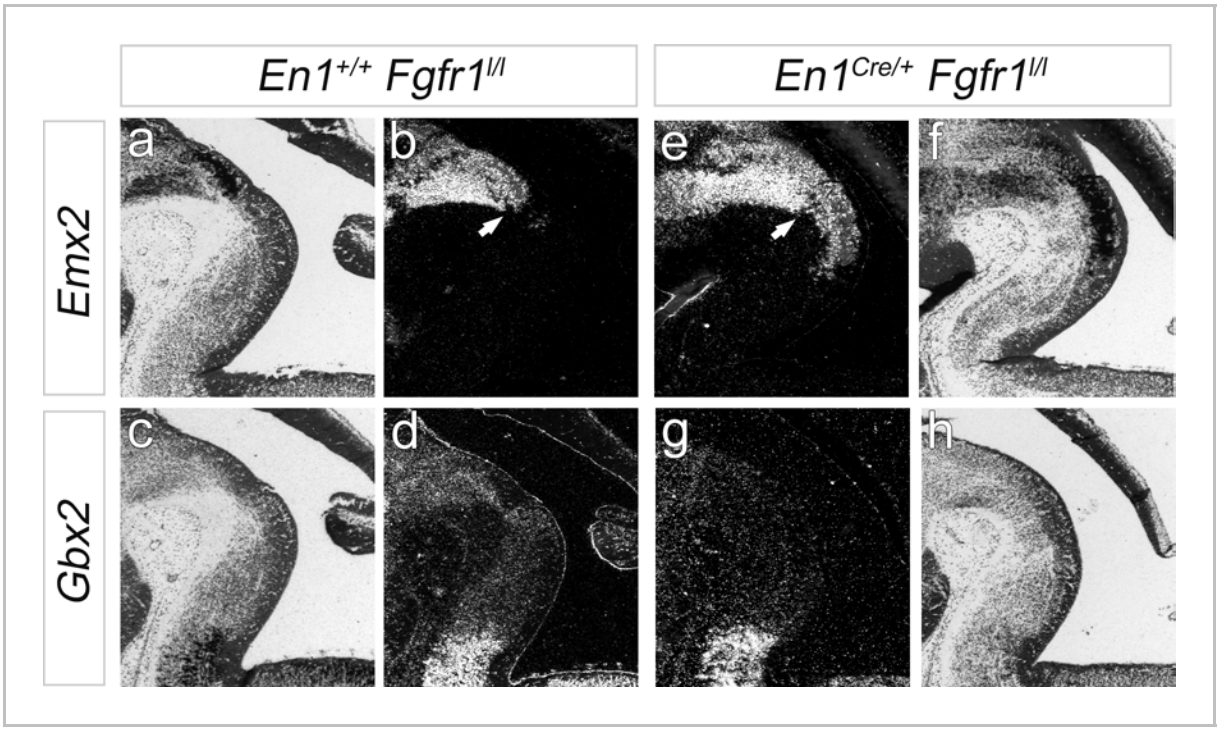

Fig. 9 Expression of *Emx2* is expanded caudally in the MHR of E13.5 *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ embryos. Wt (a-d) and mutant (e-h) embryos were cut sagittally and sections were hybridized with probes for *Emx2* (b,e) and *Gbx2* (d,g). The corresponding brightfield pictures for b,d,e and g are given in a,c,f and h, respectively. Arrows mark the caudal *Emx2* expression boundary as seen in the wt embryo.

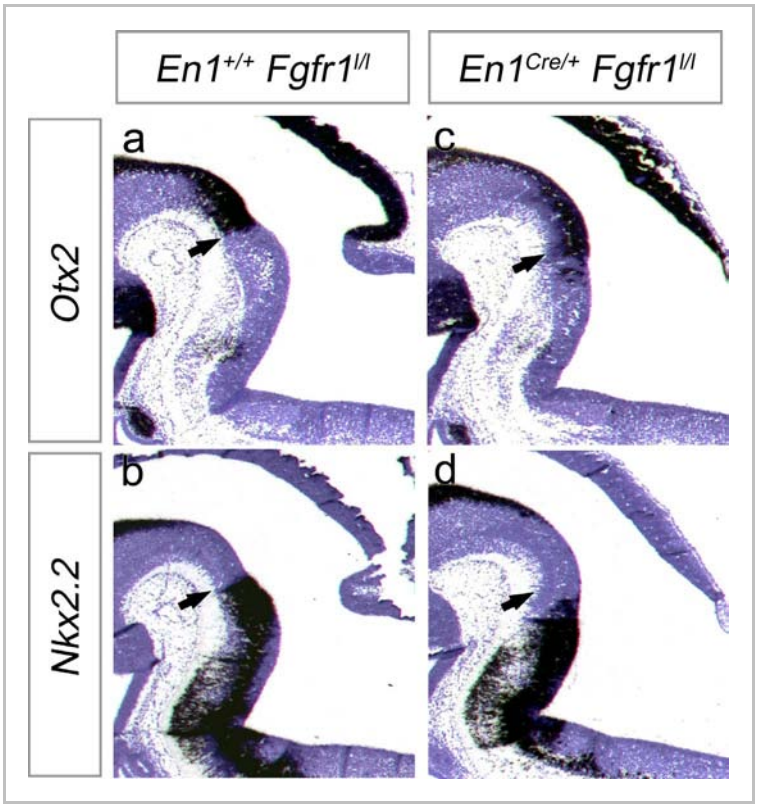

Fig. 10

Fig. 10 *Otx2* expression is expanded caudally at the expense of *Nkx2.2* expression in the ventral MHR of *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ embryos at E13.5. Pictures show brightfield images of consecutive sagittal sections through the ventral mesencephalic flexure of an wt (a,b) and an *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ (c,d) embryo with black staining for the hybridized probes. Patches of cells expressing *Otx2* (a,c) are expanded caudally, while *Nkx2.2* (b,d) expression is reduced in the anterior hindbrain. Arrows indicate the position of the wt mid-/hindbrain boundary.

Wnt1 is expressed along the ventral midbrain and as a ring anterior to the mid-/hindbrain boundary in wildtype embryos at E13.5 (Fig. 11b). *Wnt1* expression in *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ mutant embryos was disorganized when compared to the control embryos (Fig. 11b,e). Caudal to the mid-/hindbrain boundary, *Fgf8* is also expressed in a ring in wildtype embryos (Fig. 11d). In contrast to what was found at E10.5, *Fgf8* expression in the mutants was severely reduced to a small patch of cells in the ventral MHR at E13.5 (Fig. 11g).

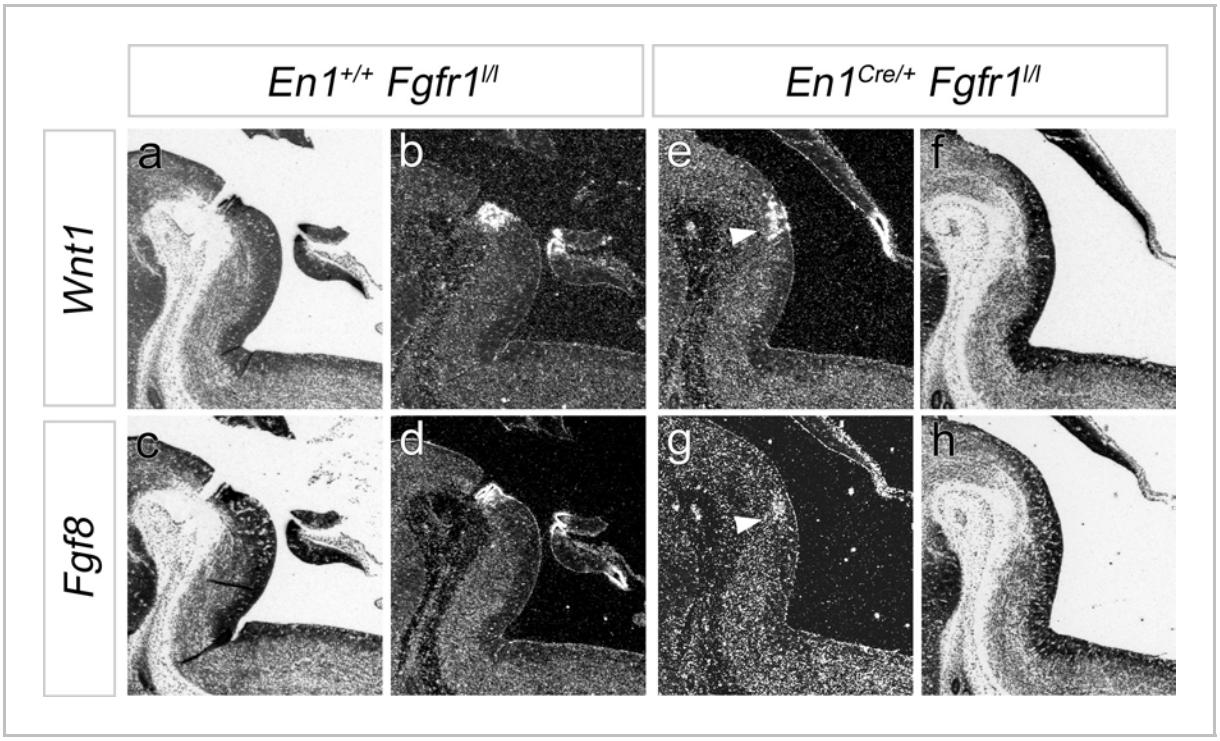

Fig. 11 *Wnt1* expression is disorganized and *Fgf8* expression is reduced in *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ embryos at E13.5. Consecutive sagittal sections of wt (a-d) and mutant (e-h) embryos were hybridized with probes for *Wnt1* (b,e) and *Fgf8* (d,g). The corresponding brightfield pictures to b,d,e and g are given in a,c,f and h, respectively. Arrowheads in e and g point to the expression domains of *Wnt1* and *Fgf8*.

In summary, the expression of the anterior patterning genes *Otx2*, *Emx2* and *Wnt1* was expanded caudally in the ventral MHR of *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ mutants from E10.5 on, whereas the expression of the patterning genes *Nkx2.2* and *Fgf8* was downregulated in the anterior hindbrain at E13.5.

2.2.3 Wnt pathway genes are expressed normally in *En1$^{Cre/+}$ Fgfr1$^{l/l}$* mutants at E13.5

Wnt1 is a growth factor that is important for proliferation, specification and differentiation of cells in the MHR, especially in the midbrain (Panhuysen et al., 2004; Prakash et al., 2006). It has been shown before that *Wnt1* expression can be induced by FGF8 and that loss of *Fgf8* in the MHR leads to the loss of other genes expressed at the mid-/hindbrain organizer, including *Wnt1* (Crossley et al., 1996; Liu et al., 1999; Martinez et al., 1999; Chi et al., 2003). These findings suggested that components of the Wnt signaling pathway might have been affected in the *En1$^{Cre/+}$ Fgfr1$^{l/l}$* mutants. Although Trokovic et al. could show expression of *Wnt1* in the MHR of *En1$^{Cre/+}$ Fgfr1$^{l/l}$* embryos at E10.5 (see also Figs. 8 and 11), maintenance of *Wnt1* expression and of other components of the Wnt pathway had not been investigated at later stages. In wildtype embryos, *Wnt1* is expressed as a small ring anterior to the MHB and in the roof and basal plate of the mesencephalon (Fig. 12b). I also detected *Wnt1* expression in the ventral and dorsal midbrain of *En1$^{Cre/+}$ Fgfr1$^{l/l}$* embryos, although the ventral *Wnt1* expression seemed less compact than in the control embryos (Fig. 12h). *Wnt5a*, another Wnt family member expressed along the ventral midbrain, the Wnt receptors *Frizzled 3* (*Fzd3*) and *Fzd6* and the *LIM homeobox transcription factor 1b* (*Lmx1b*), which is involved in induction and maintenance of *Wnt1* expression, were expressed alike in *En1$^{Cre/+}$ Fgfr1$^{l/l}$* mutants and wildtype littermates (Fig. 12c-f, i-l), confirming that, besides *Wnt1*, components of the Wnt pathway are expressed normally in *En1$^{Cre/+}$ Fgfr1$^{l/l}$* embryos at E13.5.

2.2.4 The floor and basal plate are intact in *En1$^{Cre/+}$ Fgfr1$^{l/l}$* embryos at E13.5

Dorsal tissue has been shown to be lost in *En1$^{Cre/+}$ Fgfr1$^{l/l}$* mutants. To verify that tissue in the ventral MHR is intact along the dorso-ventral (d/v) axis and that alterations in patterning are not due to a loss of the floor plate or basal plate, the expression of *Shh* and *Nkx2.2* was examined in coronal sections at E13.5. At this stage, *Shh* marks the floor plate in the hindbrain and in the midbrain also the basal plate. It was expressed normally in the ventral midbrain of mutant animals (Fig. 13b,e). *Nkx2.2* has been shown to be involved in the specification of neuronal subtypes along the d/v axis of the caudal neural tube, including serotonergic neurons (Briscoe et al., 1999; Cheng et al., 2003; Pattyn et al., 2003). In wildtype embryos it is expressed along the boundary between the basal and the alar plate (Fig. 13c). Its expression was not altered along the d/v axis of the mutant MHR (Fig. 13f).

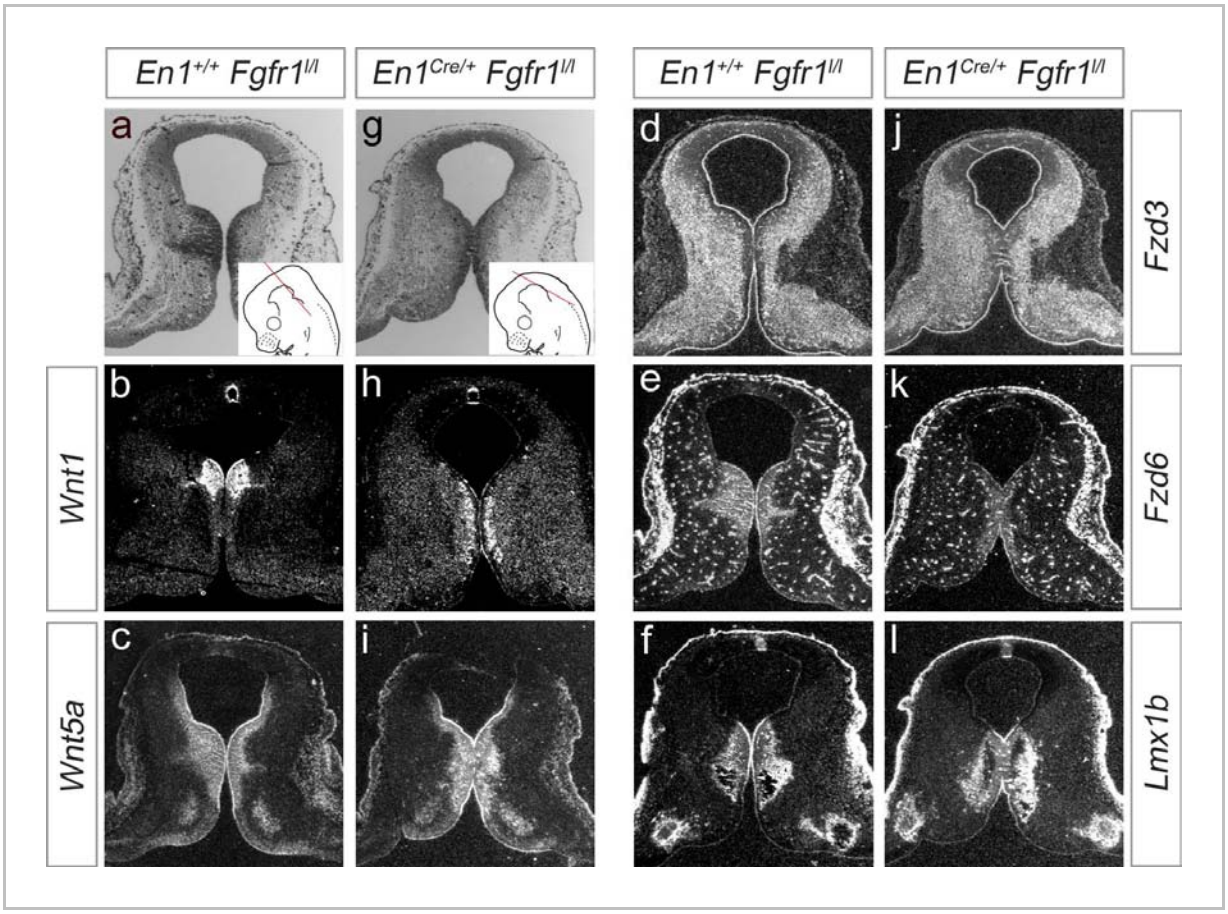

Fig. 12 Expression of genes related to the Wnt signaling pathway is not changed in E13.5 *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ embryos. Coronal sections through the midbrain of wt (a-f) and mutant (g-l) embryos were hybridized with probes for *Wnt1* (b,h), *Wnt5a* (c,i), *Fzd3* (d,j), *Fzd6* (e,k) and *Lmx1b* (f,l). Brightfield pictures of the region are given in a and g. For the orientation of the sections from control and mutant embryos compare insets in a and g.

These results confirm that the floor plate and basal plate of the mutants are intact and that no tissue is lost along the d/v axis in the ventral MHR.

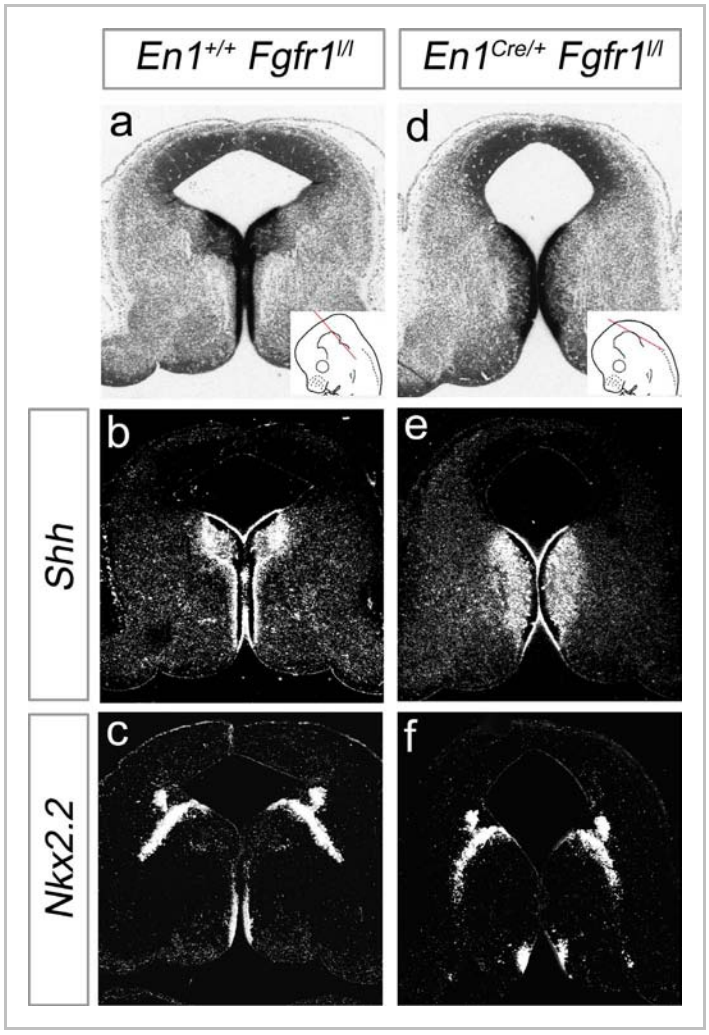

Fig. 13 The floor and basal plate are intact in *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ embryos at E13.5. Coronal sections through the posterior midbrain of control (a-c) and mutant (d-f) embryos. *Shh* (b,e) and *Nkx2.2* (c,f) are expressed with similar d/v extent in wt and *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ embryos. Corresponding brightfield pictures are given in a and d. Mutants were cut further from the top, therefore the plane of sectioning is tilted towards anterior (see insets in a and d).

2.2.5 Development of dopaminergic and serotonergic neurons is altered in *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ mice

Dopaminergic and serotonergic neurons are two prominent neuronal populations in the ventral midbrain and hindbrain, respectively, which have been shown to be induced by Fgf signaling (Ye et al., 1998). As described above, I found a caudal expansion of the expression domains of midbrain patterning genes and a downregulation of hindbrain patterning genes. These changes in patterning might have led to alterations in the specification of ventral neuronal populations. Therefore, I analyzed the expression of genes associated with the development and function of dopaminergic and serotonergic neurons in the MHR.

2.2.5.1 Dopaminergic and serotonergic precursor domains are changed in *En1$^{Cre/+}$ Fgfr1$^{l/l}$* mice at E13.5

Transcription factors which are expressed in dopaminergic and serotonergic precursor cells and neurons are robustly detectable at E13.5. *Nuclear receptor related protein 1* (*Nurr1/Nr4a2*) is expressed in the ventral midbrain, including midbrain dopaminergic precursor cells and neurons. *Lmx1b* is expressed in the ventral midbrain and hindbrain, encompassing midbrain dopaminergic and hindbrain serotonergic precursor cells and neurons. One factor specifically expressed in differentiating midbrain dopaminergic neurons is the *Paired like homeodomain transcription factor 3* (*Pitx3*), while *Pet1* is expressed in differentiating serotonergic neurons. I analyzed the expression of these genes in E13.5 control and *En1$^{Cre/+}$ Fgfr1$^{l/l}$* embryos to detect changes in the differentiation of midbrain dopaminergic and hindbrain serotonergic neurons.

In sagittal sections of E13.5 *En1$^{Cre/+}$ Fgfr1$^{l/l}$* embryos I found a caudal expansion of *Nurr1* and *Pitx3* expression, as compared to the control littermates. The caudal boundary of both expression domains was diffuse and not as compact as in the wildtype (Fig. 14b,c,g,h). The expression of *Lmx1b* was broader in the anterior r1 of *En1$^{Cre/+}$ Fgfr1$^{l/l}$* mutants, but the position of its caudal expression boundary was the same as in the control embryos (Fig. 14d,i). Together with the caudal expansion of the *Pitx3* and *Nurr1* expression domains, an obvious reduction in the a/p extent of the *Pet1* expression domain was observed in r1 of the mutant embryos (Fig. 14e,j) at E13.5. These results show that transcription factors expressed in midbrain dopaminergic precursor cells and neurons are expanded caudally, whereas *Pet1*, which is expressed in serotonergic precursor cells and neurons, is reduced in the ventral MHR.

2.2.5.2 Dopaminergic neurons are expanded caudally in *En1$^{Cre/+}$ Fgfr1$^{l/l}$* embryos at E13.5

At E13.5, terminally differentiated dopaminergic and serotonergic neurons are also present in the ventral MHR and markers for functionally differentiated mDA and 5-HT neurons can be studied. Terminally differentiated midbrain dopaminergic neurons are characterized by the combined expression of *Pitx3* and *Dopamine reuptake transporter* (*Dat*), whereas differentiated serotonergic neurons express *Pet1* and *Serotonin transporter* (*Sert)*. I analyzed the expression of the mDA associated transcription factor *Pitx3* and of the functional marker gene *Dat* in comparison to the functional 5-HT neuron marker *Sert*, to see whether the changes observed in the expression of transcription factors in mDA and 5-HT precursors and

presumptive neurons were reflected in the expression of functional markers for these neuronal populations.

In sagittal sections at E13.5, the expression domain of *Pitx3* in the ventral mesencephalic flexure of control embryos extended from the middle of the mesencephalic flexure to the isthmic constriction (Fig. 15b). *Dat* expression was embedded in the *Pitx3* expression domain and also terminated at the isthmic constriction (Fig. 15c). Caudally adjacent to the expression borders of *Pitx3* and *Dat*, *Sert* expression extended as a stripe in anterior r1 (Fig. 15d). In *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ embryos the expression domains of *Pitx3* and *Dat* were less compact and were expanded caudally in comparison to control littermates (Fig. 15f,g). To the same extent to which the caudal border of *Pitx3* and *Dat* expression was expanded, *Sert* expression in the anterior r1 was lost in the mutant embryo. Therefore, the expression of functional markers for mDA and 5-HT neurons reflects the caudal expansion of mDA and the anterior reduction of 5-HT associated transcription factors.

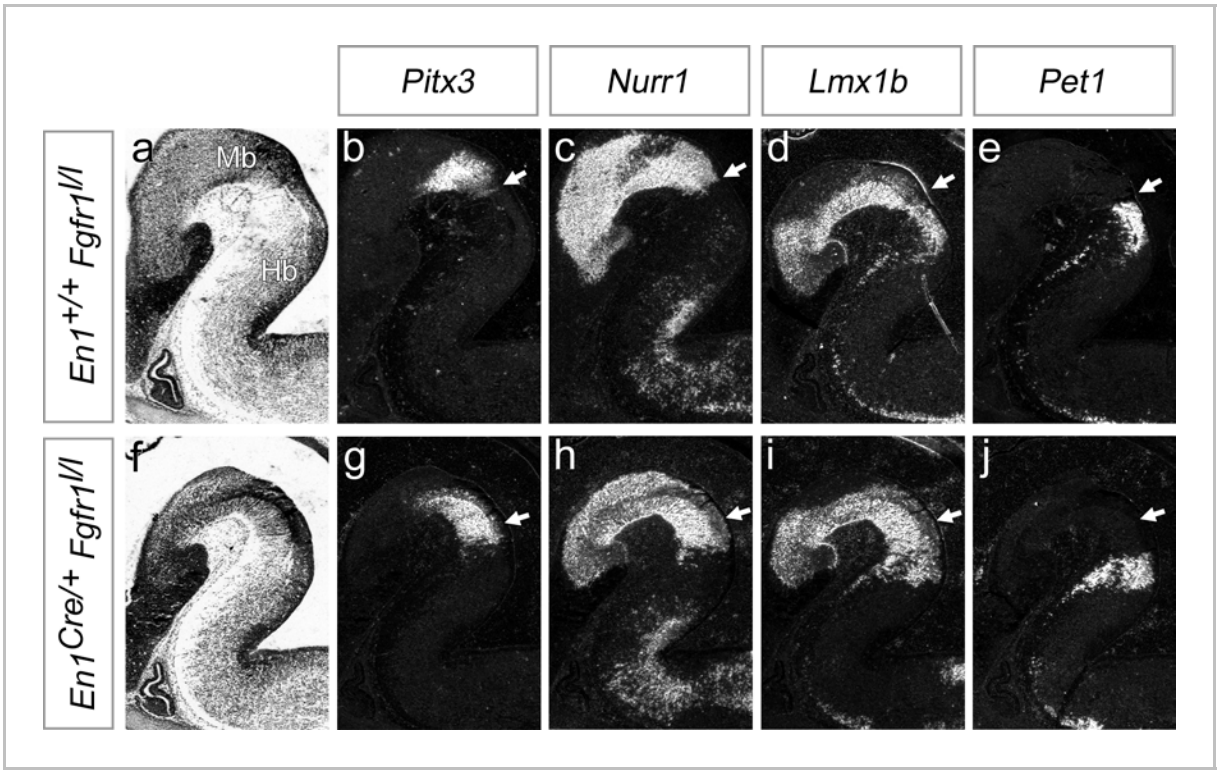

Fig. 14 Expression of transcription factors associated with midbrain dopaminergic precursors and neurons is expanded caudally at the expense of the serotonergic transcription factor *Pet1* in *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ embryos at E13.5. Consecutive sagittal sections of control (a-e) and *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ (f-j) embryos were hybridized with probes for *Pitx3* (b,g), *Nurr1* (c,h), *Lmx1b* (d,i) and *Pet1* (e,j). The caudal boundary of the *Pitx3* and *Nurr1* expression domains is diffuse and expanded caudally in mutants (b,c,g,h) whereas anterior expression of *Pet1* is lost (e,j). *Lmx1b* expression is comparable in control and mutant embryos (d,i). Pictures in a and f are bright field pictures of the mesencephalic flexure. Mb: midbrain, Hb: hindbrain. Arrows indicate the position that corresponds to the wildtype mid-/hindbrain boundary.

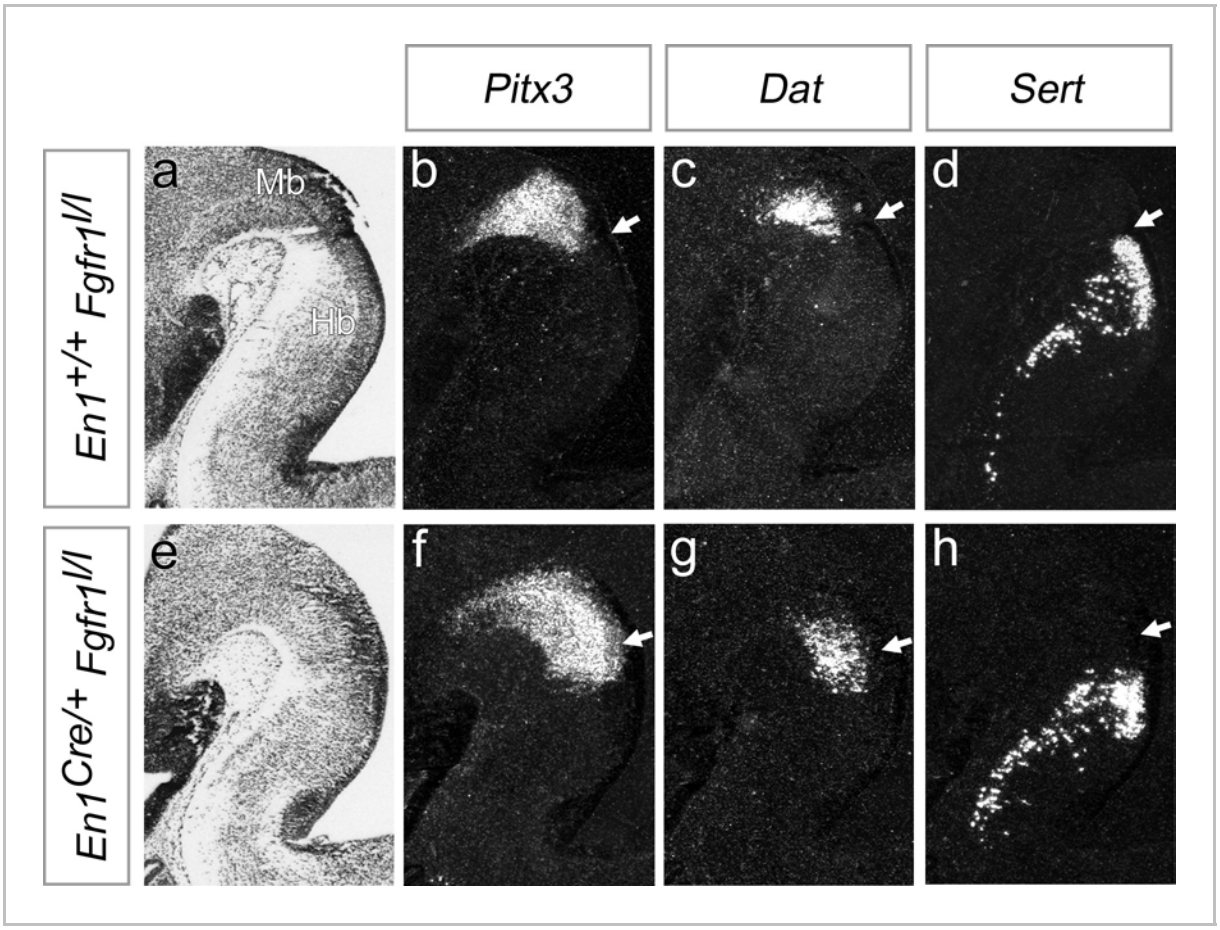

Fig. 15 Midbrain dopaminergic neurons are expanded caudally at the expense of anterior serotonergic neurons in the MHR of *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ embryos at E13.5. Sagittal sections of the ventral mesencephalic flexure of a E13.5 wt (a-) and mutant (e-h) embryo. Brightfield pictures in a and e correspond to the sections in b and f. Expression of *Pitx3* (b,f) and *Dat* (c,g) was expanded caudally and more diffuse in the ventral MHR of *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ mutants. *Sert* expression was lost in the anterior hindbrain of the mutant (d,h). Mb: midbrain, Hb: hindbrain. Arrows point to the position that corresponds to the wildtype mid-/hindbrain boundary.

2.2.5.3 Serotonergic neurons are reduced within an *Otx2* positive domain in *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ embryos at E13.5

Mature serotonergic neurons express *Pet1* and *Sert*. Sagittal sections of the mesencephalic flexure were hybridized with probes for *Otx2*, *Pet1* and *Sert* to analyze the serotonergic neurons in the anterior hindbrain in relation to a patterning gene that was expanded caudally in *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ mutants. In control embryos, *Otx2* expression showed a sharp boundary at the isthmus (Fig. 16b). Expression of *Pet1* (Fig. 16c) and *Sert* (Fig. 16d) caudally abutted the *Otx2* expression boundary. In *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ embryos, *Otx2* expression was expanded into the anterior hindbrain and showed a speckled caudal pattern (Fig. 16f), as shown before.

Interestingly, in the mutants, the anterior expression of *Pet1* (Fig. 16g) and *Sert* (Fig. 16h) was completely absent and no patches of serotonergic neurons were found in the region in which speckled expression of *Otx2* was detected. Horizontal and coronal sections at E13.5 confirmed the absence of *Sert* expression in the most anterior hindbrain (data not shown).

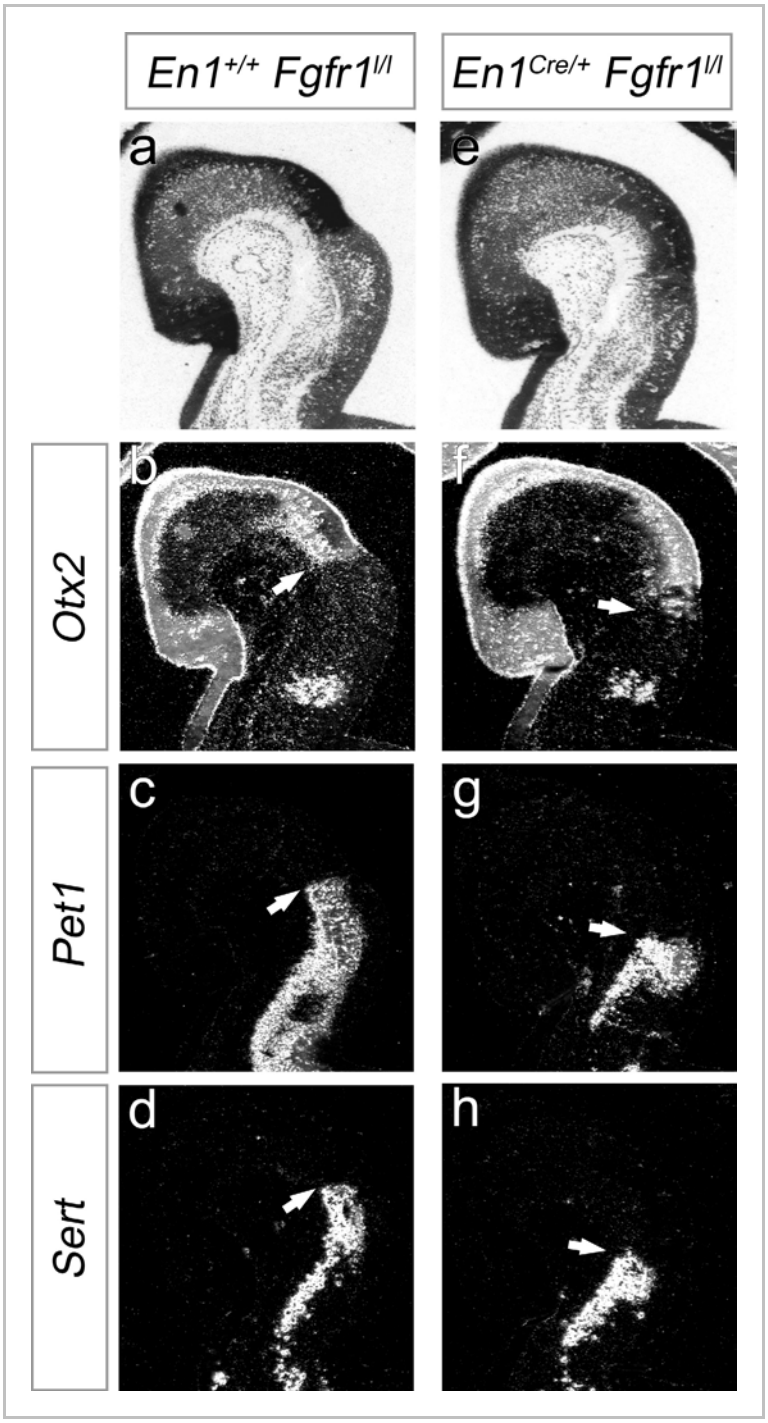

Fig. 16 Serotonergic neurons are reduced in an *Otx2* positive domain in anterior hindbrain of *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ embryos at E13.5. Sagittal sections of wt (a-d) and mutant (e-h) mesencephalic flexure and anterior hindbrain. Brightfield pictures in a and e correspond to darkfield pictures of *Otx2* probe hybridization in b and f. Consecutive sections hybridized with *Pet1* (c,g) and *Sert* (d,h) probe show a loss of the most anterior serotonergic neurons in the mutants in the area in which *Otx2* is

expanded. MF: mesencephalic flexure, Mb: midbrain, Hb: hindbrain. Arrows indicate the caudal expression boundary of Otx2.

2.2.6 Not all serotonergic neurons in rhombomere 1 depend on Fgfr1

If all serotonergic neurons in the anterior hindbrain depended on the function of Fgfr1, they should have been lost throughout the domain in which *Fgfr1* was inactivated. To find out whether the loss of *Sert* expression in the most anterior hindbrain was a direct consequence of the inactivation of *Fgfr1* in the same domain, I compared the expression domains of the homeodomain transcription factor *En1*, which is expressed across the mid-/hindbrain boundary and had been used to drive the expression of the *Cre recombinase*, *Fgfr1* and *Sert* in control and $En1^{Cre/+}$ $Fgfr1^{l/l}$ embryos at E13.5.

Comparison of expression on consecutive sections of wildtype embryos showed, that the *Sert* expression in anterior r1 was included within the *En1* expression domain (Fig. 17a). Expression of *Fgfr1* extended throughout the MHR (although it was strongest in the ventricular zone of the neuroepithelium) and accompanied *Sert* expression in the anterior r1 (Fig. 17b). In the $En1^{Cre/+}$ $Fgfr1^{l/l}$ mutant, the remaining part of the *Sert* expression domain in anterior r1 was still located within the *En1* expression domain (Fig. 17c). Consequently, it was also located within the area in which *Fgfr1* was inactivated (Fig. 17d). This showed that only a part of the serotonergic neurons that arise in anterior r1 is lost in the domain in which *Fgfr1* is inactivated in the mutants. Therefore, the remaining serotonergic neurons in anterior r1 do not directly depend on Fgfr1.

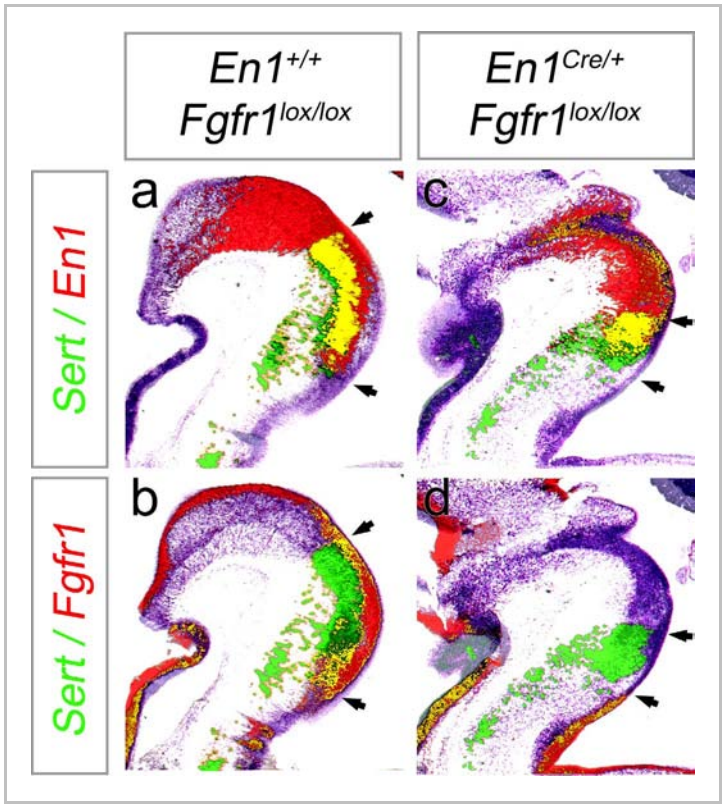

Fig. 17 Not all serotonergic neurons in anterior r1 depend on Fgfr1. False color overlays of consecutive sagittal sections from a wt (a,b) and a mutant (c,d) E12.5 embryo, which were hybridized with probes for *En1* (red in a,c), *Fgfr1* (red in b,d) and *Sert* (green in a-d), are shown. Yellow color marks regions with an overlap of expression. In the wt, anterior serotonergic neurons are embedded within *En1* expression (a) and are accompanied by expression of *Fgfr1* (b). In the mutant embryo, *Sert* expression is reduced, but is also embedded within the *En1* expression domain (c) and is located within the area in which *Fgfr1* expression is lost (d). Arrows demarcate the borders of *Sert* expression in anterior r1.

2.2.7 Changes in dopaminergic and serotonergic neurons are specific and persistent in *En1$^{Cre/+}$ Fgfr1$^{l/l}$* mice

The analysis of patterning factors, dopaminergic and serotonergic precursor markers and markers for differentiated dopaminergic and serotonergic neurons in the embryonic MHR suggested an caudal expansion of midbrain patterning and of midbrain dopaminergic neurons. On the other hand, patterning molecules in the anterior hindbrain were reduced and the anterior serotonergic neurons in the hindbrain were absent. These changes in embryonic development might have led to persistent alterations in the dopaminergic and serotonergic systems at later time points. To determine whether the changes in the dopaminergic and serotonergic populations of the MHR were conserved at later stages, I analyzed expression of dopaminergic and serotonergic markers at P0 and in adult *En1$^{Cre/+}$ Fgfr1$^{l/l}$* mice.

2.2.7.1 Dopaminergic neurons are absent from the VTA of *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ mice at P0

The part of the dopaminergic system that forms the ventral tegmental area (VTA), in the ventral midline of the midbrain, is located between the laterally adjacent halves of the substantia nigra pars compacta (SNpc) in newborn wildtype animals (Fig. 18b,c). *TH*, *Dat* and *Pitx3*, markers for mature midbrain dopaminergic neurons, were not expressed in the midline of the ventral midbrain in *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ mutants (Fig. 18e,f and data not shown). Nevertheless, expression of all three marker genes was found in the SNpc, with a gap in between these domains, where the VTA is located in the control littermates (Fig. 18b,c,e,f and data not shown). Thus, dopaminergic neurons were absent from the VTA of *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ mutants at P0. However, Nissl staining of the same sections did not suggest a loss of tissue in the ventral midline of the midbrain (Fig.18a,d).

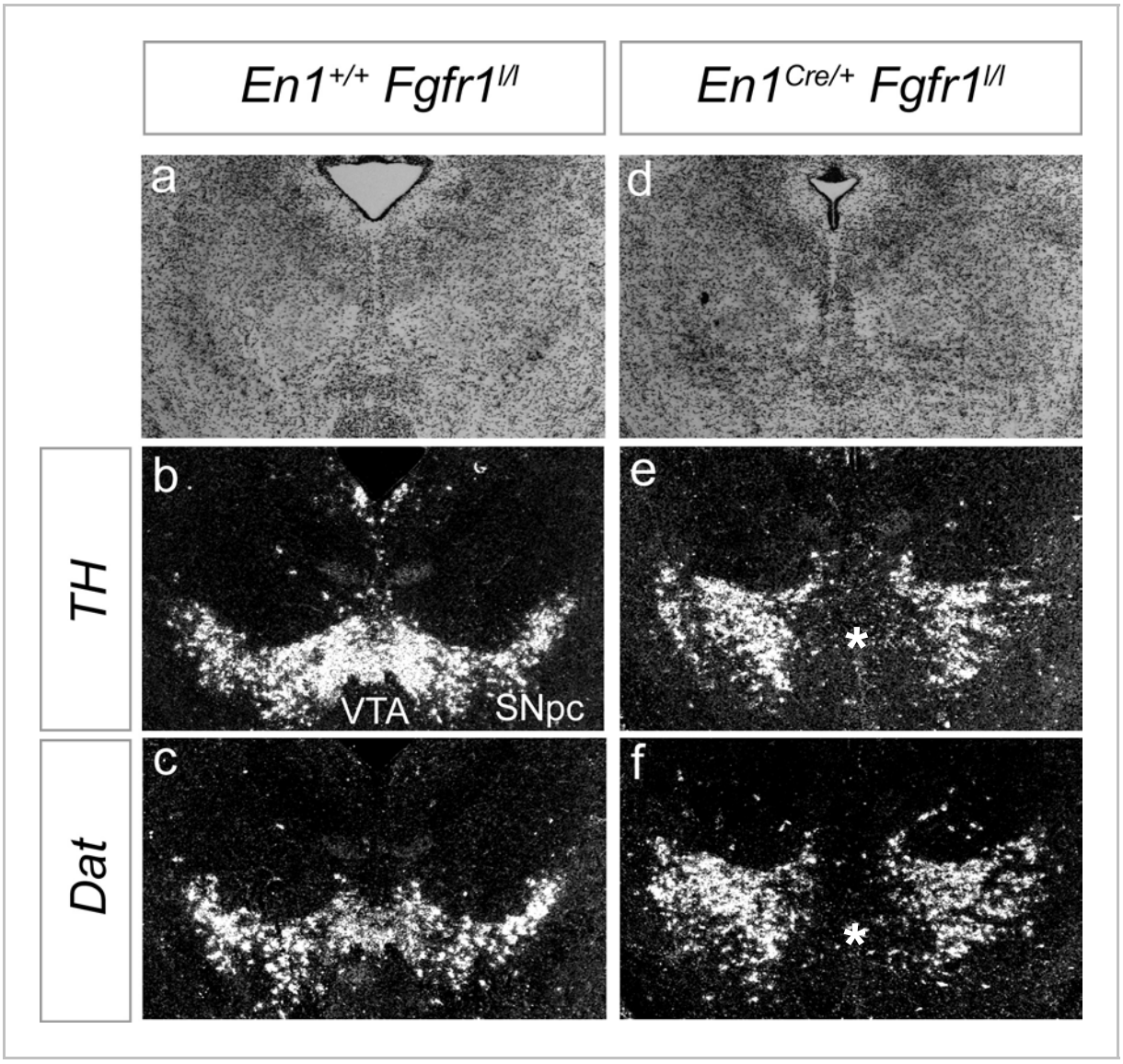

Fig. 18

Fig. 18 Dopaminergic neurons are absent from the VTA of *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ mice at P0. Coronal sections of a wt (a-c) and an *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ (d-f) P0 brain, hybridized with probes for *TH* (b,e) and *Dat* (c,f). Expression of *TH* and *Dat* is absent from the VTA in the mutant animals (e,f). Brightfield pictures of the region are shown in a and d. VTA: ventral tegmental area, SNpc: substantia nigra pars compacta. Asterisks highlight the area free of dopaminergic marker expression in the mutant brain.

2.2.7.2 Dopaminergic neurons are absent from the medial VTA but found in the anterior hindbrain of adult *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ mice

The absence of dopaminergic neurons from the VTA of *En1*$^{Cre/+}$ *Fgfr1l*$^{/l}$ mice at P0 was puzzling, as dopaminergic neurons seemed to be expanded into the hindbrain at E13.5 (Fig. 15). Therefore, I decided to determine the number and distribution of TH positive cells in the MHR of adult mutants, to find out whether they were reduced in number or whether some were located in another position, away from the VTA. Immunohistochemistry for TH on horizontal sections from adult brains was used for these purposes. In horizontal sections of adult *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ animals, the number of TH and Dat positive cells in the medial ventral midbrain (i.e., in the medial VTA) was obviously reduced. Instead, scattered TH and Dat reactive cells were found posterior to the dopaminergic cells of the SNpc in the mutants (Fig. 19Aa,b and data not shown). However, stereological analysis of the number of TH positive cells in the SNpc and VTA, including the scattered cells in the most anterior hindbrain of mutant animals, revealed no change in the total number of TH positive neurons in this region (Fig. 19B; average number of TH positive cells was 23290 ± 608 in control and 23678 ± 2401 in mutant animals; n = 5 for each genotype, p = 0.88 in t-test).

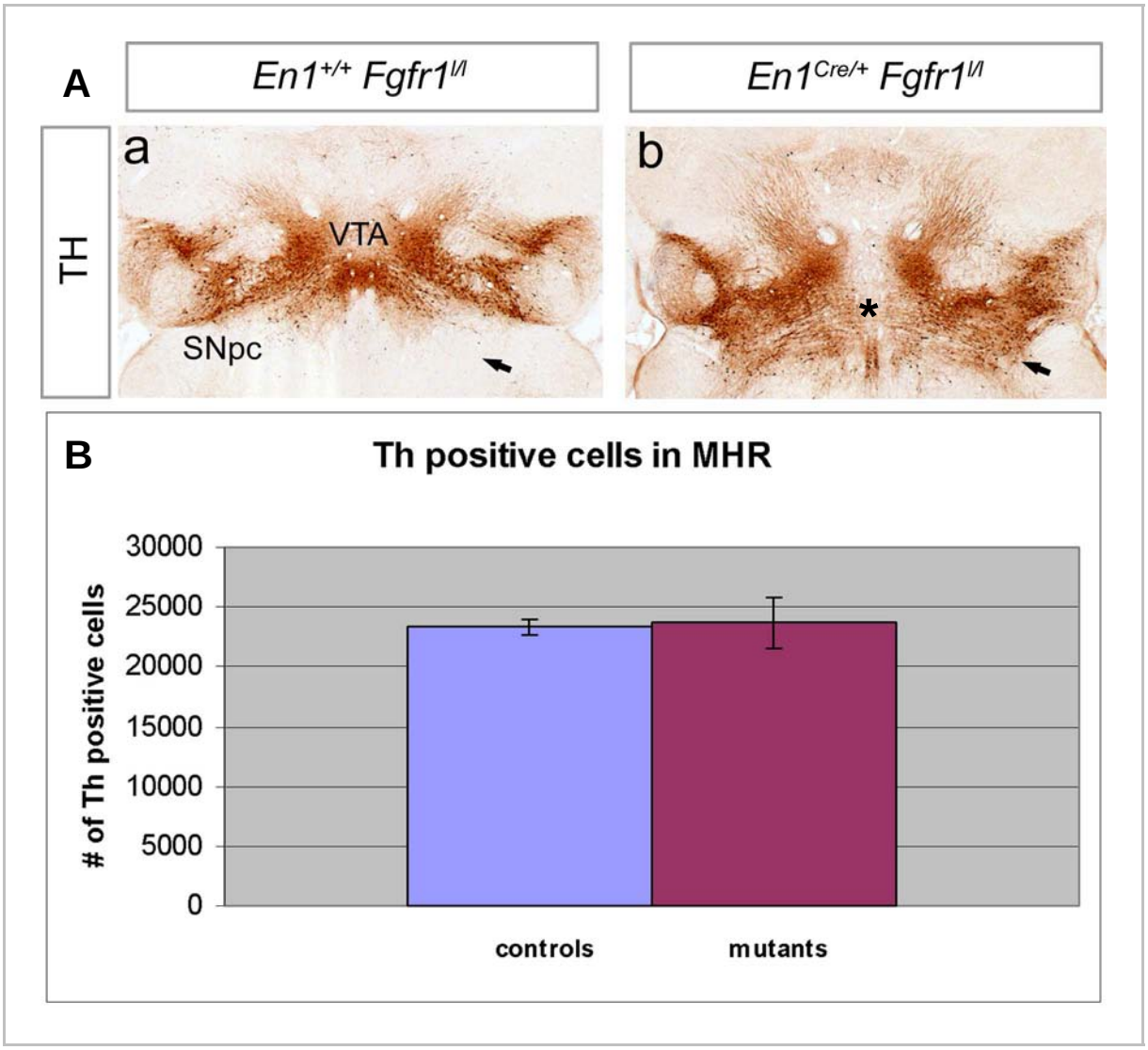

Fig. 19 Dopaminergic neurons are present in normal numbers, but are absent from the medial VTA and found in the anterior hindbrain of adult *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ mutants. A Horizontal sections through the ventral midbrain of a wt (a) and an *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ (b) adult brain immunostained using an antibody against TH. No TH reactive cells are found in the medial VTA, but ectopic TH reactive cells are present in the anterior hindbrain of the mutant (b). SNpc: substantia nigra pars compacta, VTA: ventral tegmental area. Asterisk in b marks the TH negative area in the mutant VTA, arrows indicate the region where ectopic TH positive cells are found in the mutant. **B** The number of TH positive cells is comparable in the MHR of control and mutant mice.

2.2.7.3 Ectopic Calbindin and Girk2 positive cells in the anterior hindbrain of *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ mice

While dopaminergic neurons were absent from the medial VTA of adult *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ mutants, the total number of dopaminergic neurons in the MHR was not changed. The presence of ectopic dopaminergic neurons in the anterior hindbrain suggested a defect in migration of VTA dopaminergic neurons. Dopaminergic neurons of the medial VTA have

been shown to express the calcium binding protein Calbindin but not the potassium channel subunit G-protein coupled inward rectifying potassium channel 2 (Girk2), which is preferentially expressed in dopaminergic neurons of the substantia nigra (Liang et al., 1996; Schein et al., 1998; Thompson et al., 2005). To elucidate the identity of the ectopic DA in the anterior hindbrain of the *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ mutants, I performed immunohistochemistry for Calbindin and Girk2 in relation to TH. Calbindin is expressed in TH positive cells in the VTA and in a small subpopulation of cells expressing TH in the SN of control mice (Fig. 20a,b). In the mutants, expression of Calbindin was found caudal to the SN (Fig. 20d), in the same region in which ectopic TH protein was detected (Fig. 20e). Girk2 protein is found in TH positive cells of the SN and in a few cells in the lateral VTA (Fig. 20b,c). In the *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ mutant, Girk2 protein was also found in the ectopic TH positive cells caudal to the SN (Fig. 20e,f). These findings suggest that the ectopic DA neurons in the anterior hindbrain of the *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ mutant animals are midbrain DA neurons, although a definitive VTA character of these ectopic TH positive cells could not be confirmed.

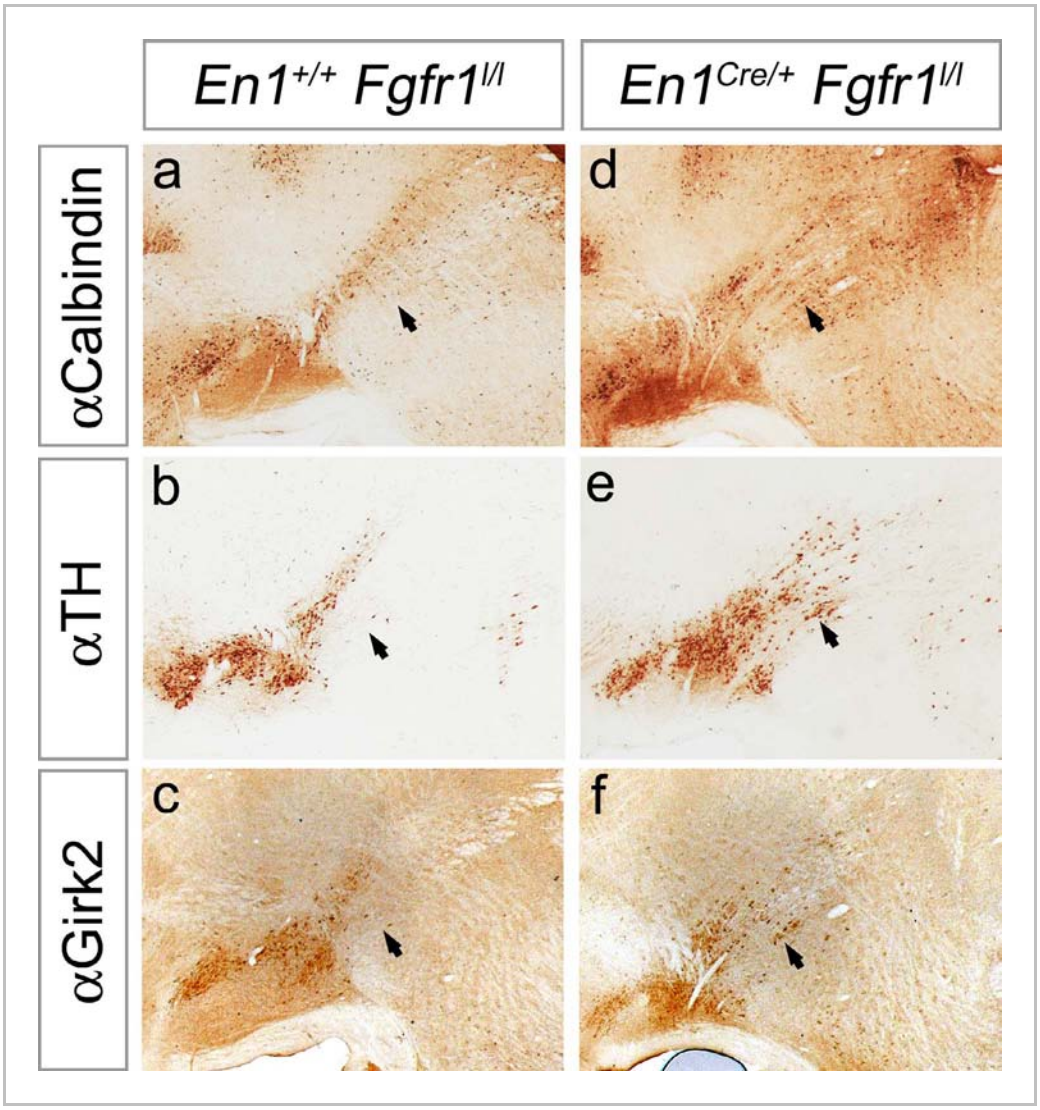

Fig. 20

Fig. 20 Ectopic calbindin positive cells are found together with TH positive cells caudal to the SN in *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ mutants. Staining with antibodies against Calbindin (a,c) and TH (b,d) in sagittal sections from the ventral mid-/hindbrain region of a wt (a,b) and a mutant adult brain. Anterior is to the left. While the expression of Calbindin and TH in the wt is condensed in the dopaminergic neurons of the substantia nigra and VTA (a,b), the anterior hindbrain of the mutant harbors ectopic patches of cells expressing calbindin (c) and TH (d). Arrows point to the area in the anterior hindbrain in which ectopic calbindin and TH positive cells are found in the mutant.

2.2.7.4 Reduction of serotonergic neurons in anterior rhombomere 1 of *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ mice is persistent at P0

Analysis of dopaminergic neurons at P0 and in adult *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ animals revealed a permanent change in the position of dopaminergic neurons in the MHR. Because the serotonergic neurons were reduced at E13.5, they were also studied in the *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ mutants at P0, to see if this reduction was persistent.

The DR of P0 control animals, the most anterior population of serotonergic neurons, forms a characteristic bird shaped structure of *Sert* positive cells in coronal sections of the anterior hindbrain (Fig. 21b). This structure was lost in the DR of *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ brains and only few scattered cells showed expression of *Sert* at P0 (Fig. 21e), confirming the persistent reduction of the anterior serotonergic neurons at P0. Like in the ventral midbrain, also in the area of the DR nucleus Nissl staining did not reveal an obvious loss of tissue (Fig. 21a,d).

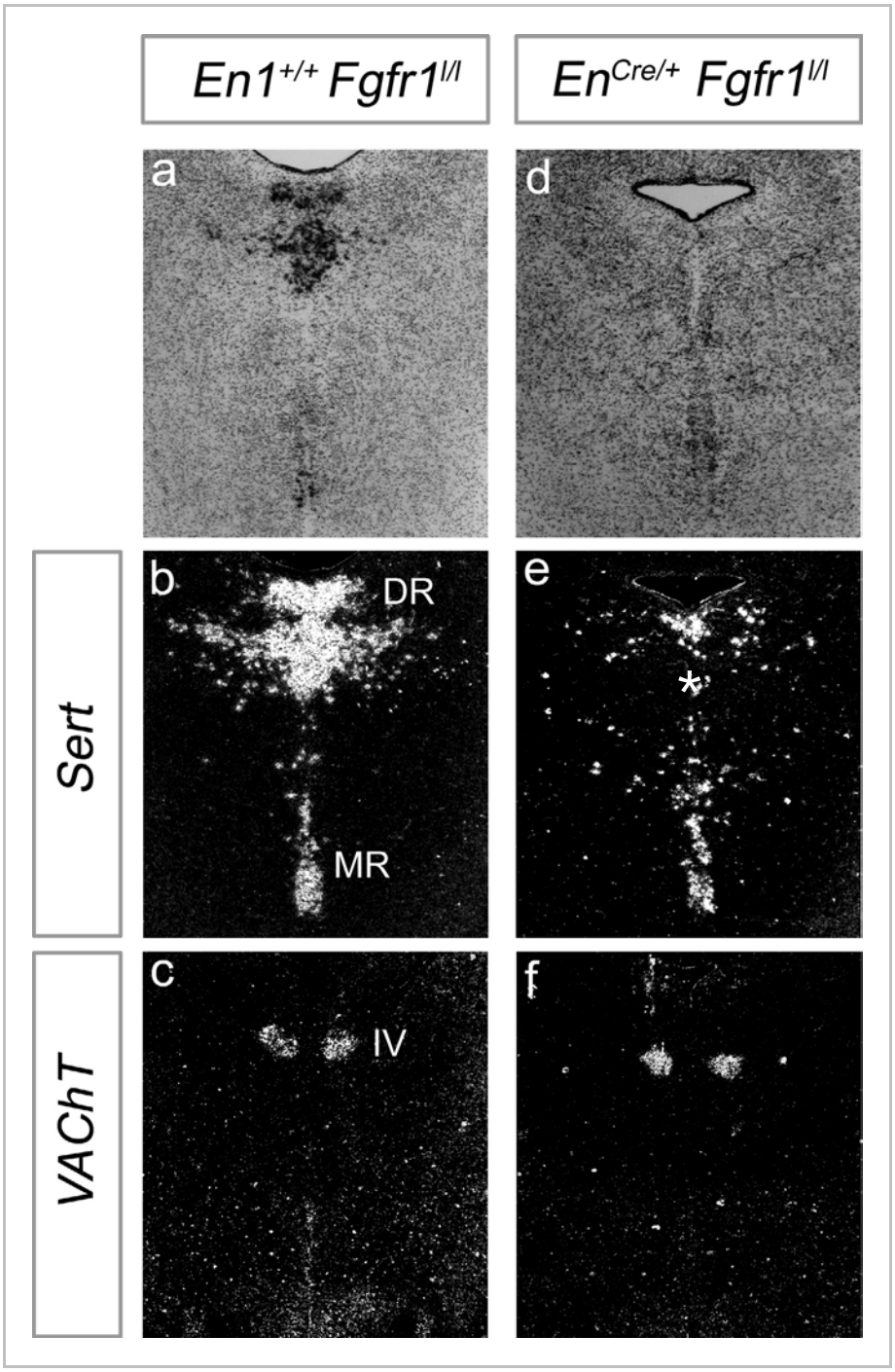

Fig. 21 Specific reduction in serotonergic neurons of the dorsal raphe in P0 *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ mutants. Coronal sections of a wt (a-c) and a mutant (d-f) P0 brain were hybridized with probes for *Sert* (b,e) and *VAChT* (c,f). Brightfield pictures of the region are shown in a and d. While serotonergic neurons in the mutant (e) were reduced in comparison to the wt (b), the cholinergic neurons of the 4th cranial nerve were not affected (c,f). DR: dorsal raphe nucleus, MR: medial raphe nucleus, IV: nucleus of 4th nerve. Asterisk marks the area in which serotonergic neurons are missing.

2.2.7.5 The number of serotonergic neurons is reduced in the anterior hindbrain of adult *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ mice

It was interesting that the reduction of serotonergic neurons at embryonic stages was persistent at P0. Nevertheless, the observed reduction might have been recovered during postnatal maturation of the brain. To see, whether a reduction of serotonergic neurons was also detectable in adults and to quantify the number of serotonergic neurons, brains from adult *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ and control animals were immunostained for the neurotransmitter serotonin (5-HT).

While serotonergic neurons of the medial raphe nucleus were detected at normal numbers (Fig. 22Ab,d), 5-HT immunoreactivity in the dorsal raphe nucleus was reduced in mutant animals (Fig. 22Aa,c). Stereological analysis revealed, that the number of 5-HT positive cells in the DR of *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ animals was reduced by 45% in average (Fig. 22B; 7872 ± 821 5-HT positive cells in wt, 4320 ± 449 in mutant anterior DR nuclei; n = 5 for each genotype, p = 0.01 in t-test). 5-HT neurons in the MR were present in comparable numbers in wt and mutant brains (Fig. 22C; 8640 ± 1914 5-HT positive cells in wt, 9072 ± 2534 in mutant caudal DR nucleus; n = 5 for each genotype, p = 0,91 in t-test). Thus, the reduction which was found in the anterior serotonergic neurons of *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ mutants at E13.5 and P0 lasted into adulthood. Parts of these studies have been published in (Jukkola et al., 2006e).

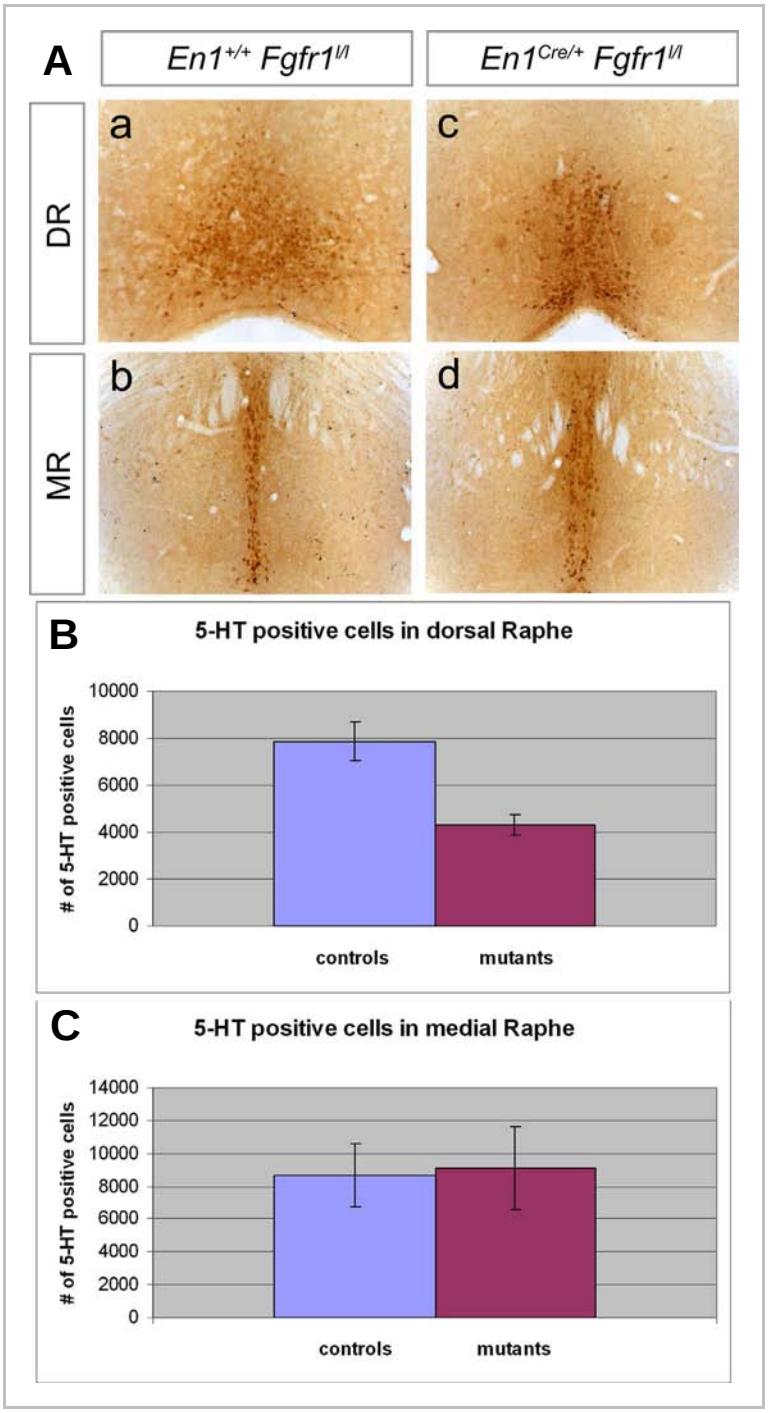

Fig. 22 Reduced number of serotonergic neurons in the DR of *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ mutants. A Immunostaining for 5-HT on horizontal sections of a wt (a,b) and an *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ (c,d) adult brain. 5-HT staining in the DR is more abundant in horizontal sections from a wt (a) than from a mutant (c) brain. In the MR, wt and mutant show similar expression of 5-HT (b,d). **B** The number of 5-HT positive cells in the DR of mutant adult brains is reduced by 45%. **C** The number of 5-HT positive cells in the MR of adult control and mutant brains is similar.

2.2.7.6 Changes in dopaminergic and serotonergic neurons are specific to these neuronal populations in *En1$^{Cre/+}$ Fgfr1$^{l/l}$* mutants

The ectopic positioning of dopaminergic neurons in the anterior hindbrain and the reduction of serotonergic neurons in the dorsal raphe nucleus are obvious phenotypes that were found in two major neuronal populations of the ventral MHR. Other neuronal cell groups might have been affected as well and the loss of the anterior serotonergic neurons might have been due to a general loss of tissue in the anterior hindbrain. I analyzed the cholinergic nuclei of the 3rd and 4th cranial nerves (i.e. nucleus oculomotorius and nucleus trochlearis), which arise early in development and are located in close vicinity to the midbrain dopaminergic neurons of the VTA and the serotonergic neurons of the DR respectively, to see if in *En1$^{Cre/+}$ Fgfr1$^{l/l}$* mutants neighbouring neuronal populations are changed.

In P0 wildtype and *En1$^{Cre/+}$ Fgfr1$^{l/l}$* mutant mice, the cholinergic neurons in the nucleus of the 4th cranial nerve were visualized by hybridization with a probe for the *Vesicular acetylcholine transporter* (*VAChT*) (Fig. 21c,f) in parallel to the serotonergic neurons. The neurons of the 4th cranial nerve arise immediately adjacent to the serotonergic neurons of the dorsal raphe nucleus in the anterior hindbrain. They were therefore used to find out whether the loss of neurons was specific to the DR serotonergic neurons. Indeed, the expression of *VAChT* was similar in control (Fig. 21c) and *En1$^{Cre/+}$ Fgfr1$^{l/l}$* (Fig. 21f) mice, suggesting that specifically serotonergic neurons were affected in the anterior hindbrain of the mutants at P0. In adult *En1$^{Cre/+}$ Fgfr1$^{l/l}$* and control brains the nuclei of the 3rd and 4th cranial nerve were visualized by immunostaining for parvalbumin, a calcium binding protein that is expressed in the neurons of these nuclei. Although the 4th cranial nerve was less compact and the distinction between the 3rd and 4th cranial nerves was less clear in mutant brains than in wildtype brains, both were present in adult *En1$^{Cre/+}$ Fgfr1$^{l/l}$* mutants (Fig. 23Aa,b). Furthermore, the size of the 4th cranial nerve was not altered in the mutants as compared to wildtype littermates (Fig. 23B; average area of 4th nerve was 42344 ± 10166 µm^2 in control and 45833 ± 8865 µm^2 in mutant animals; n = 4 for wt, n = 3 for mutant, p = 0.95 in t-test). This suggests that there is no general loss of tissue in the anterior hindbrain of *En1$^{Cre/+}$ Fgfr1$^{l/l}$* mutants and that neighbouring neuronal populations are not affected in the same way as the midbrain dopaminergic and anterior hindbrain serotonergic neurons.

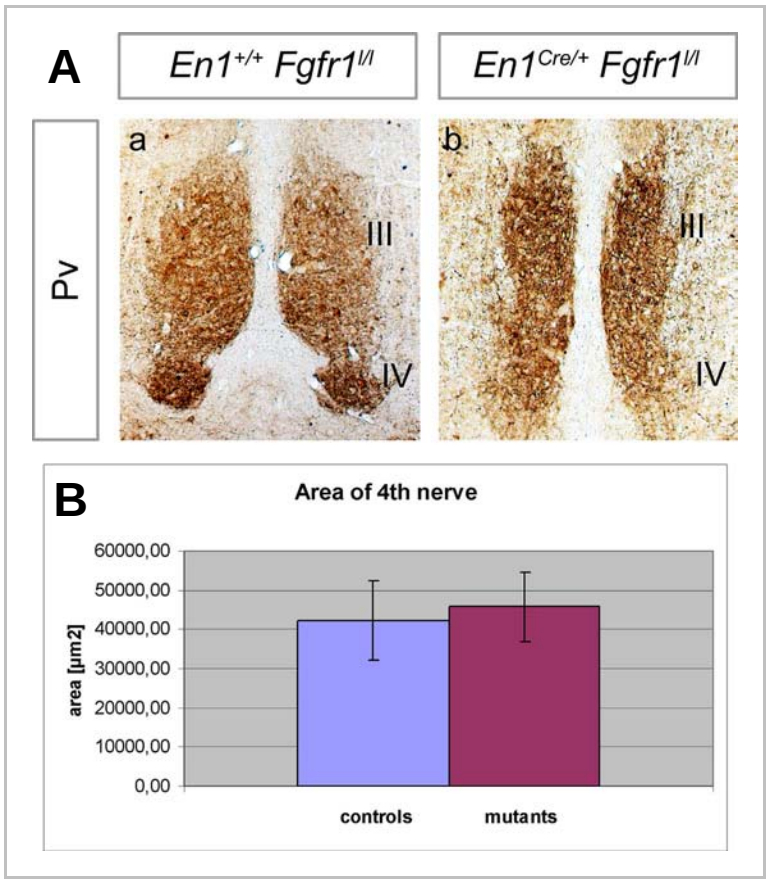

Fig. 23 The 3rd and 4th cranial nerve are present in adult *En1Cre/+ Fgfr1l/l* brains. A anti-Parvalbumin immunostaining on horizontal sections through the MHR of an *En1+/+* Fgfr1*l/l* and an *En1Cre/+ Fgfr1l/l* adult brain show that the nuclei of the 3rd and 4th cranial nerve are present in the mutant (Aa,b). However, the distinction between both nuclei is not as clear in the mutant as in the wt. III: nucleus of 3rd nerve (nucleus oculomotorius), IV: nucleus of 4th nerve (nucleus trochlearis). **B** The area of the 4th nerve is not altered in *En1Cre/+ Fgfr1l/l* mutants.

On the basis of these results it was assumed that alterations in the ventral MHR of adult *En1Cre/+ Fgfr1l/l* mutants are restricted to the dopaminergic neurons of the VTA and the serotonergic neurons of the anterior DR. These changes arise early in development and are persistent into adulthood. Parts of these studies have been published (Jukkola et al., 2006d).

2.2.8 Fgf signaling is active in the ventral MHR of *En1Cre/+ Fgfr1l/l* mutants until E13.5

While *Fgf8* conditional MHR mutant mice lost all tissues in the MHR (Chi et al., 2003), the *En1Cre/+ Fgfr1l/l* mutant mice only lost the IC and cerebellar vermis in the dorsal MHR and no loss of tissue was apparent in the ventral MHR (Trokovic et al., 2003). This suggested that Fgf signaling is still active in the ventral MHR of *En1Cre/+ Fgfr1l/l* mutant embryos. On the other

hand, the patterning defects and changes in the VTA and DR of the *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ mutant mice must have been a consequence of the loss of *Fgfr1* expression and might have affected Fgf signaling in the MHR of the mutant animals. I therefore analyzed the expression of Fgf signaling targets in the MHR of *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ mutant embryos.

2.2.8.1 Fgf signaling is active in the ventral MHR of *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ mutants at E10.5

Sef1, Sprouty1 and MKP3 are inhibitors and direct transcriptional targets of Fgf signaling. To determine whether Fgf signaling is active in the ventral MHR after inactivation of *Fgfr1*, I analyzed their expression in the ventral MHR at E10.5. All three genes are expressed in a broad ring around the MHB in control embryos (Fig. 24b-d). No expression of these genes could be detected in the dorsal MHR of *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ mutants (Fig. 24f-h). However, *Sef1*, *Spry1* and *MKP3* were all expressed in the ventral MHR of *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ embryos at E10.5 (Fig. 24f-h).

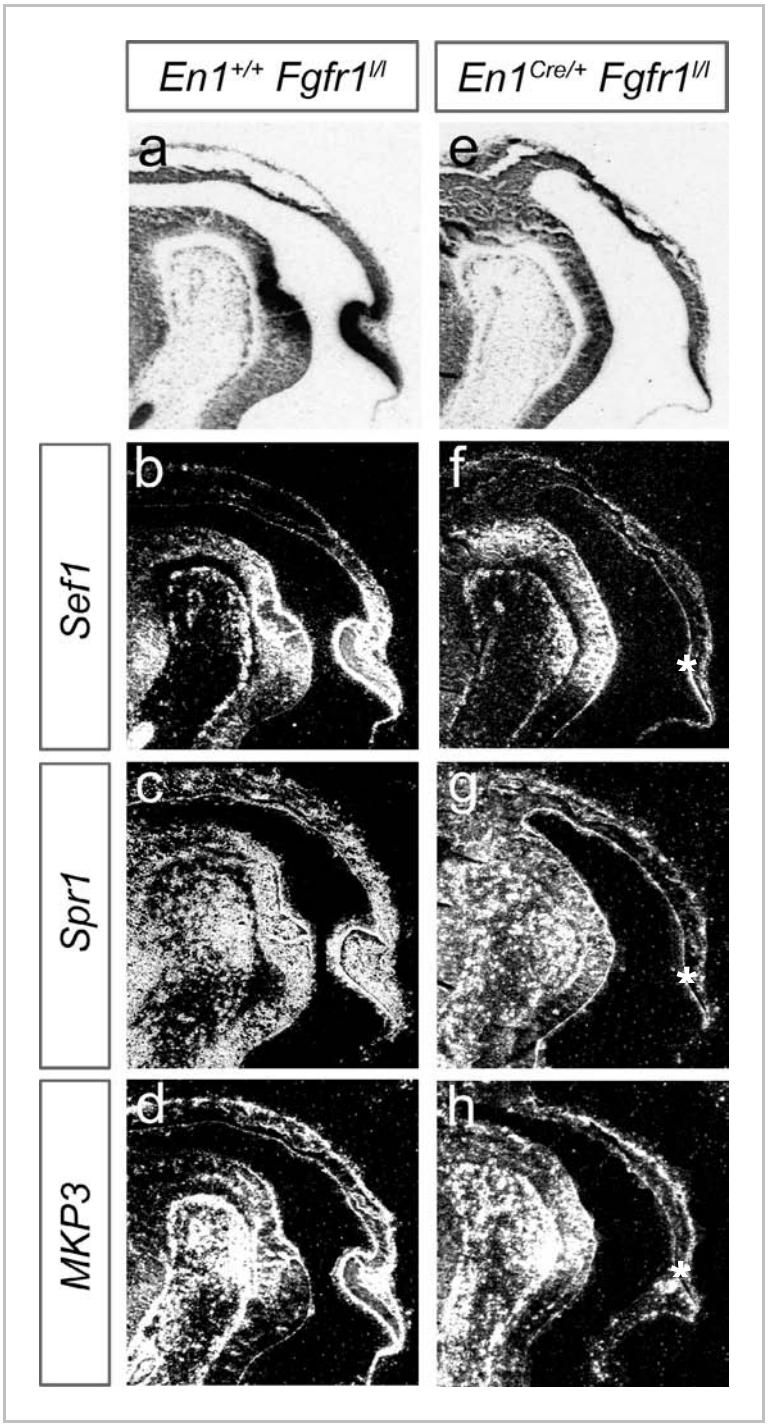

Fig. 24 Direct targets of Fgf signaling are expressed in the ventral MHR of *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ embryos at E10.5. Sagittal sections of the MHR of control (a-d) and mutant (e-h) embryos hybridized with probes for *Sef1* (b,f), *Spr1* (c,g) and *MKP3* (d,h). All three Fgf target genes are expressed in the ventral and dorsal MHR of the control embryos (b-d). In mutant embryos, the ventral MHR shows expression of *Sef1* (f), *Spr1* (g) and *MKP3* (h), whereas the dorsal region is free of Fgf target gene expression. Brightfield pictures of the region are given in a and e. Asterisks mark lack of signal in the dorsal MHR of mutant embryos.

2.2.8.2 Targets of Fgf signaling are expressed in the ventral MHR of *En1$^{Cre/+}$ Fgfr1$^{l/l}$* embryos at E13.5

Although Fgf signaling was still active at E10.5, when early patterning defects in the ventral MHR were detected, it might not have been maintained until E13.5, when alterations in the dopaminergic and serotonergic neurons were first observed. Therefore, I studied the expression of *Sef1* and *MKP3* in *En1$^{Cre/+}$ Fgfr1$^{l/l}$* mutants at E13.5. In wildtype embryos, *Sef1* and *MKP3* were both expressed across the mid-/hindbrain boundary (Fig. 25b,c). In *En1$^{Cre/+}$ Fgfr1$^{l/l}$* embryos, expression of *Sef1* and *MKP3* was absent from the dorsal but was still detected in the ventral MHR (Fig. 25e,f). These findings suggested that Fgf signaling is active in the ventral MHR of *En1$^{Cre/+}$ Fgfr1$^{l/l}$* mutants at least until E13.5.

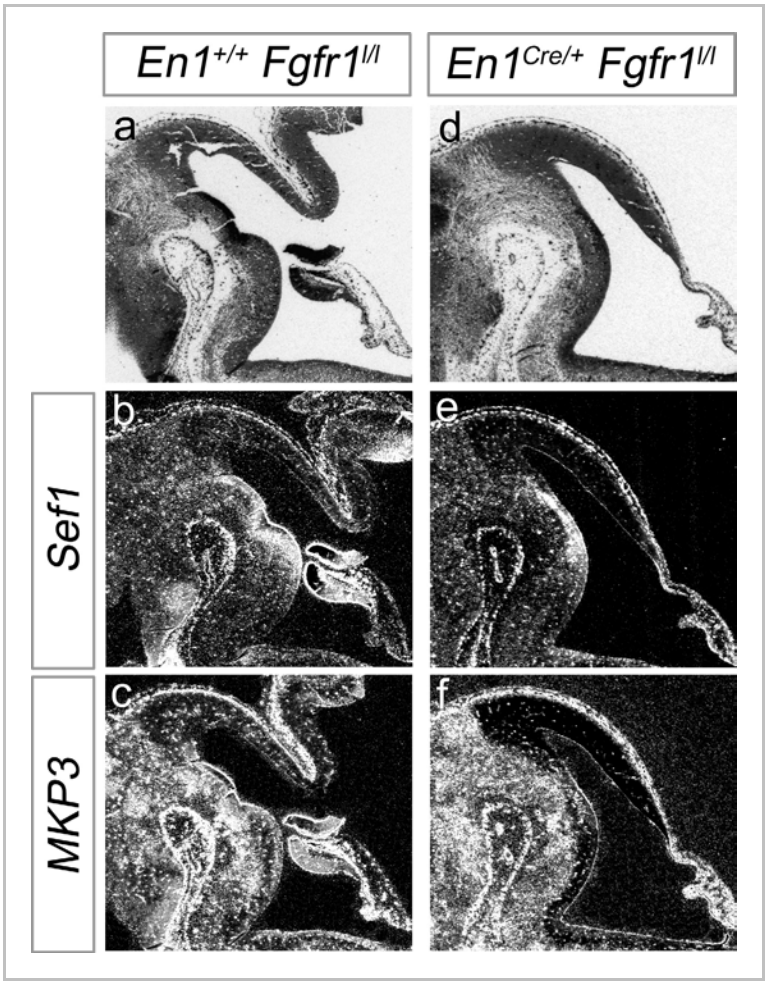

Fig. 25 Fgf signaling targets are expressed in the ventral MHR of *En1$^{Cre/+}$ Fgfr1$^{l/l}$* embryos at E13.5. Sagittal sections of the mid-/hindbrain region of control (a-c) and mutant (d-f) embryos hybridized with probes for *Sef1* and *MKP3* showed expression of both genes in the ventral and dorsal MHR of wt (b,c) embryos. In mutant embryos, both Fgf target genes were still expressed in the ventral MHR at E13.5 (e,f).

In summary, Fgf signaling is still active in the ventral MHR of *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ mutants and the phenotypic alterations observed demonstrate a specific requirement of Fgfr1 for normal development of dopaminergic and serotonergic neurons in the ventral MHR.

2.2.9 Expression of Fgf receptors in the MHR of early mouse embryos

The *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ mice did not show a loss of tissue in the ventral MHR, which had been described for a conditional knock out of *Fgf8* in the same region (Trokovic et al., 2003; Chi et al., 2003). Furthermore, target genes of Fgf signaling were still expressed in the ventral MHR of the conditional *Fgfr1* knock out mice. A plausible explanation for the discrepancy between the phenotypes of the *Fgf8* and *Fgfr1* conditional MHR knock out mice and for the expression of Fgf target genes would be that another Fgf receptor is expressed in the ventral MHR which can mediate Fgf signaling and rescue morphogenesis of the ventral MHR. Therefore, a detailed study of Fgf receptor expression in the developing mouse MHR was carried out with special emphasis on ventral structures. As we did not find *Fgfr4* to be expressed in the MHR, we focused on the expression of *Fgf receptors 1-3*. To relate the expression of the *Fgf receptors* to other genes that are expressed in the MHR, we hybridized consecutive sections with probes for *En1*, which is expressed across the mid-/hindbrain boundary in the caudal midbrain and anterior hindbrain, and for Fgf8, which is expressed caudal to the mid-/hindbrain boundary in the anterior hindbrain from E8.5 on.

Fgfr1 had been shown to be expressed throughout the MHR before (Ishibashi and McMahon, 2002; Trokovic et al., 2003; Liu et al., 2003). We confirmed ubiquitous expression of *Fgfr1* in the MHR from stages E8.5 to E12.5, with strong expression in mesenchyme and weak expression in the neuroepithelium (Blak et al., 2005). At all time points analyzed, *Fgfr1* expression overlapped with the expression domains of *En1* and *Fgf8* (Blak et al., 2005).

Initial expression of *Fgfr2* in the midbrain at E8.5 overlapped with the anterior half of the *En1* expression domain. Caudally, *Fgfr2* expression abutted the *En1* expression in the hindbrain, leaving a gap in *Fgfr2* expression in the anterior hindbrain (Fig. 26Ab,c,d). The overlap of *Fgfr2* expression with the anterior half of the expression domain of *En1* suggested that *Fgfr2* is expressed in the entire midbrain at this stage. Indeed, the anterior expression of *Fgfr2* was abutting the expression domain of *Fgf8* at the mid-/hindbrain boundary at E8.5 (Fig. 26Af,g,h). In the hindbrain, the posterior *Fgfr2* expression domain did not reach the caudal boundary of *Fgf8* expression and did not overlap with the *En1* expression domain, meaning that *Fgfr2* is not expressed in the anterior rhombomere 1 at E8.5 (Fig. 26Af,g,h). From E9.5 on, expression of *Fgfr2* at the mid-/hindbrain boundary was passing through the ventral gap in *Fgf8* expression and was continuous along the a/p axis in the floor plate (Fig. 26Bb,c,d). In

the basal plate, the *Fgfr2* expression domain in the midbrain as well as in the hindbrain was in contact with the *Fgf8* expression at the mid-/hindbrain organizer (Blak et al., 2005; see figure 28). The alar and roof plate of the posterior midbrain and anterior hindbrain were free of *Fgfr2* transcripts at E9.5 (Blak et al., 2005; see figure 28). This dorsal gap in *Fgfr2* expression became smaller during development, until at E12.5 only the caudal part of the anlage of the IC and the most anterior part of the cerebellar anlage, where *Fgf8* was expressed, were devoid of *Fgfr2* (Fig. 26Cb,c,d).

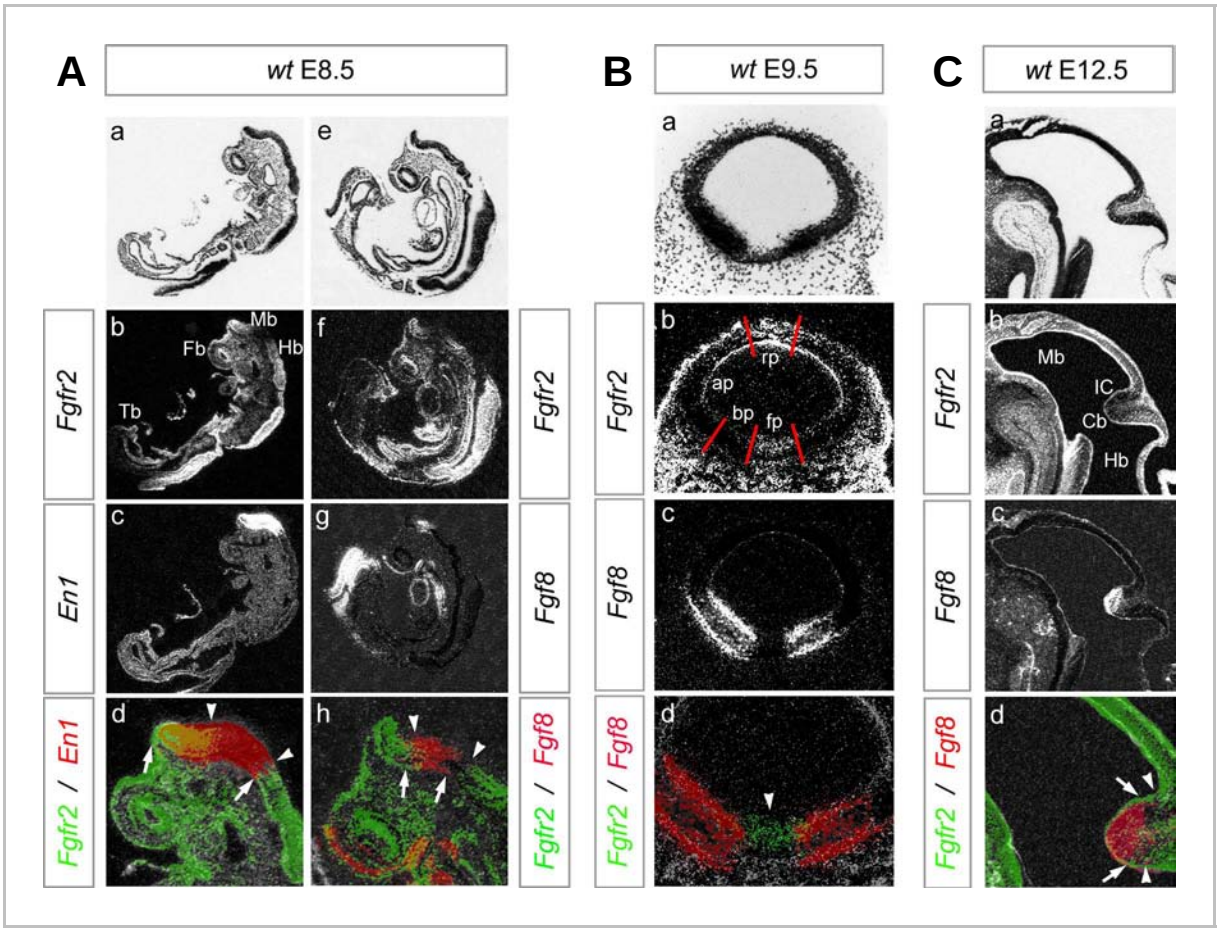

Fig. 26 *Fgfr2* is expressed in the ventral midbrain from E8.5 on and in the ventral anterior r1 after E8.5. A Sagittal sections of the E8.5 embryos shown in a and e, hybridized with probes for *Fgfr2* (b,f), *En1* (c) and *Fgf8* (g). False colored overlays from the head region of consecutive sections show expression of *Fgfr2* (green) overlapping with *En1* (red) in the midbrain (d). *Fgfr2* expression anteriorly abutted *Fgf8* (red) expression (h), but was not found in the anterior hindbrain. Arrows mark borders of expression domains given in red, arrowheads mark borders of green expression domains. **B** Consecutive coronal sections through the isthmic region of an E9.5 embryo (a-c). Expression of *Fgfr2* (b) and *Fgf8* (c) are superimposed in false colors (green and red, respectively) in d. *Fgfr2* was expressed in the floor plate of the MHR, also in the ventral gap of *Fgf8* expression at the MHB. Arrowhead in Bd points to expression of *Fgfr2* in the floor plate of the MHB. **C** Consecutive sagittal sections through the MHR of an E12.5 embryo (a-c) hybridized with probes for *Fgfr2* (b) and *Fgf8* (c). An overlay of *Fgfr2* (green) and *Fgf8* (red) expression is shown in d. Expression of *Fgfr2* is continuous

in the floor plate of the MHR. Only the anlage of the IC and the anterior cerebellum, where *Fgf8* is expressed, are free of *Fgfr2* expression. Fb: forebrain, Mb: midbrain, Hb: hindbrain, Tb: tail bud, rp: roof plate, ap: alar plate, bp: basal plate, fp: floor plate, IC: anlage of inferior colliculus, Cb: cerebellar anlage. Arrows mark borders of expression domains given in red, arrowheads mark borders of green expression domains.

In contrast to *Fgfr2*, which was expressed in the caudal midbrain from E8.5 on, *Fgfr3* expression was absent from this region at E8.5. Expression of *Fgfr3* did not overlap with *En1* expression, but abutted it anteriorly in the diencephalon and posteriorly in the hindbrain (Fig. 27Ab,c,d). In accordance with this, caudal expression of *Fgfr3* abutted the *Fgf8* expression domain in the hindbrain, but the anterior expression of *Fgfr3* in the diencephalon did not reach the *Fgf8* expression at this stage (Fig. 27Af,g,h). Thus, at E8.5 *Fgfr3* was expressed in the diencephalon and in the hindbrain, except for the most anterior part of r1. From E9.5 on, *Fgfr3* expression in the ventral neural tube drew closer to the isthmus, until at E12.5 a gap of only a few cell diameters was free of *Fgfr3* expression in the ventral midbrain, just anterior to the isthmic *Fgf8* expression (Fig. 27Bb,c,d). The caudal *Fgfr3* expression domain even showed an overlap with expression of *Fgf8* in the ventral r1 (Fig. 27Bd). In dorsal parts of the MHR, *Fgfr3* displayed a large gap at stages E9.5 - E12.5, such that *Fgfr3* was not found to be expressed in the dorsal posterior midbrain and in the dorsal hindbrain between r1 and r7 (Fig. 27Bb).

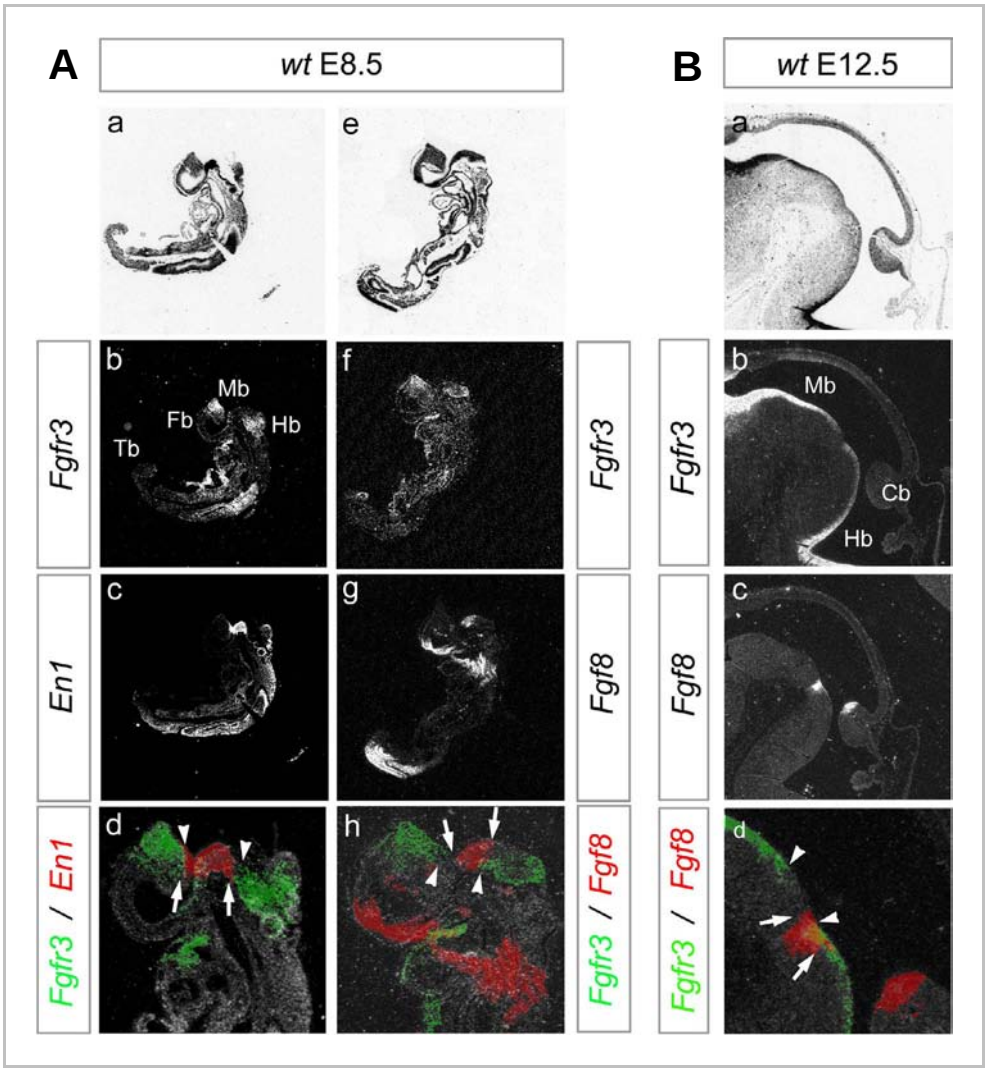

Fig. 27 *Fgfr3* is expressed in the ventral hindbrain from E8.5 on and in the ventral midbrain at E12.5. A Consecutive sagittal sections of E8.5 embryos (a-c, e-g). Sections were hybridized with probes for *Fgfr3* (b,f), *En1* (c) or *Fgf8* (g). False color overlays in d and h show expression of *Fgfr3* (green) and *En1* or *Fgf8* (red) in the head region. *Fgfr3* expression abuts *En1* in the midbrain and anterior hindbrain and Fgf8 in the hindbrain at E8.5 **B** Consecutive sagittal sections of the MHR of an E12.5 embryo (a-c). Sections were labelled with probes for *Fgfr3* (b) and *Fgf8* (c). Overlay (d) of close ups from b and c shows expression of *Fgfr3* (green) in relation to *Fgf8* (red) expression at the ventral mid-/hindbrain boundary. Fb: forebrain, Mb: midbrain, Hb: hindbrain, Tb: tail bud, Cb: cerebellar anlage. Arrows mark borders of red expression domains, while arrowheads mark borders of expression domains in green.

Taken together, our expression study on the *Fgf receptors 1-3* in early mouse development showed early expression of *Fgfr2* in the ventral MHR and of *Fgfr3* in the ventral hindbrain. Furthermore, continuous expression of *Fgfr2* in the floor plate of the MHR has not been described before. These results, together with the incomplete loss of the MHR in the $En1^{Cre/+}$ $Fgfr1^{l/l}$ mutants, suggested a possible redundancy of the Fgf receptors 1-3 in development of

the ventral MHR. The above findings were published in Developmental Dynamics (Blak et al., 2005) and are summarized in Fig. 28.

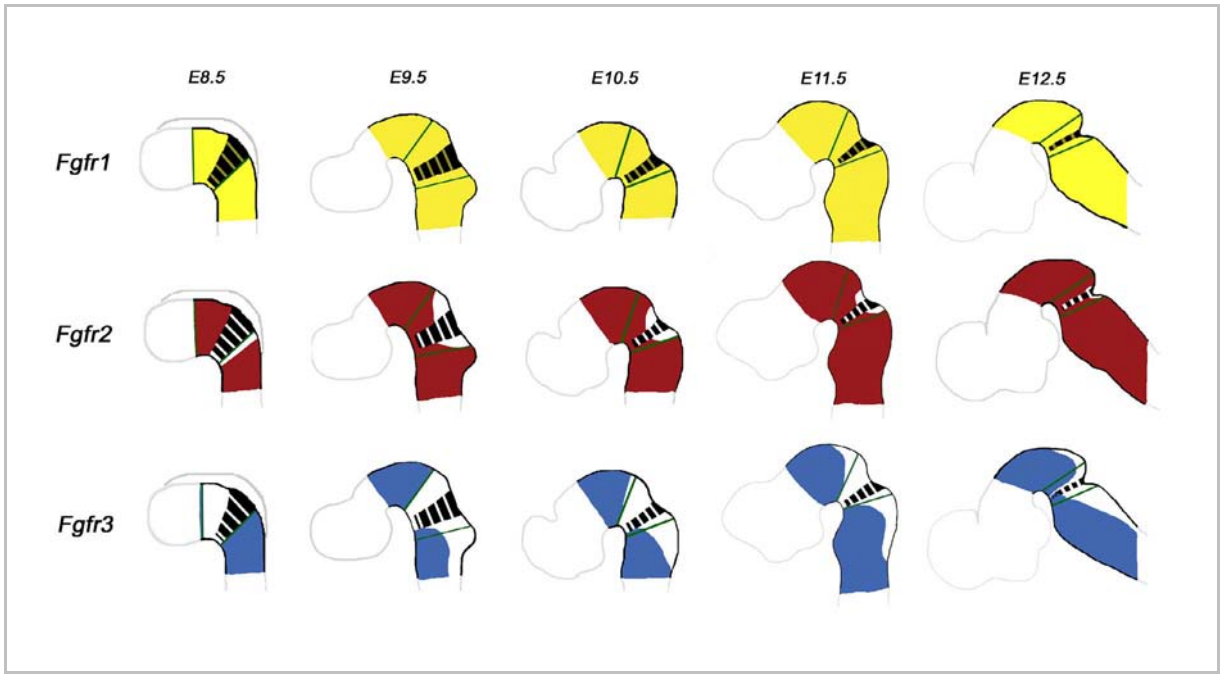

Fig. 28 Schematic overview of the expression of *Fgfr1-Fgfr3* in the mid-/hindbrain region of mouse embryos from stage E8.5 to E12.5. Expression of *Fgfr1* (yellow), *Fgfr2* (red) and *Fgfr3* (blue) is shown as a lateral view on the neural tube of mouse embryos at the stages indicated. The *Fgf8* expression domain is depicted by the black striped area, the boundaries of the *En1* expression domain are marked by green lines. Rostral is to the left.

2.2.10 *Expression of Fgfr2* and *Fgfr3* is unchanged in the ventral MHR of *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ mutants at E10.5

The expression of *Fgfr2* and *Fgfr3* in the ventral MHR of wildtype mouse embryos suggested an explanation for the activation of Fgf signaling targets in the ventral MHR of *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ mutant embryos. A logical consequence of the loss of *Fgfr1* might have been a compensatory transcriptional upregulation of the expression of *Fgfr2* and/or *Fgfr3* in the MHR of *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ mutant mice. To see whether such an upregulation took place, I analyzed the expression of *Fgfr2* and *Fgfr3* in the MHR of *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ mutant embryos at E10.5.
I found expression of *Fgfr2* throughout the floor plate of control and *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ embryos (Fig. 29b,f). In the lateral neural tube there was a gap in *Fgfr2* expression just caudal to the MHB in the ventral neural tube and in the anlage of the inferior colliculus and cerebellum in the dorsal neural tube (Fig. 29c,g) of control as well as *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ embryos. Ventral expression of *Fgfr3* in the mutant was also comparable to the wildtype controls, leaving a

gap in expression anterior to the mid-/hindbrain organizer (Fig. 29d,h). Dorsally, there was a large gap in *Fgfr3* expression in wildtype and mutant embryos (Fig. 29d,h). In all sections analyzed the intensity of the staining was roughly the same, for *Fgfr2* and *Fgfr3*, when compared between the wildtypes and the *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ mutants. Thus, normal expression of *Fgfr2* and *Fgfr3* was found in the ventral MHR of wildtype and *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ mutant embryos at E10.5. Nevertheless, the expression of *Fgfr2* in the ventral MHR of *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ mice was confirmed and might explain the activation of Fgf target genes in this region.

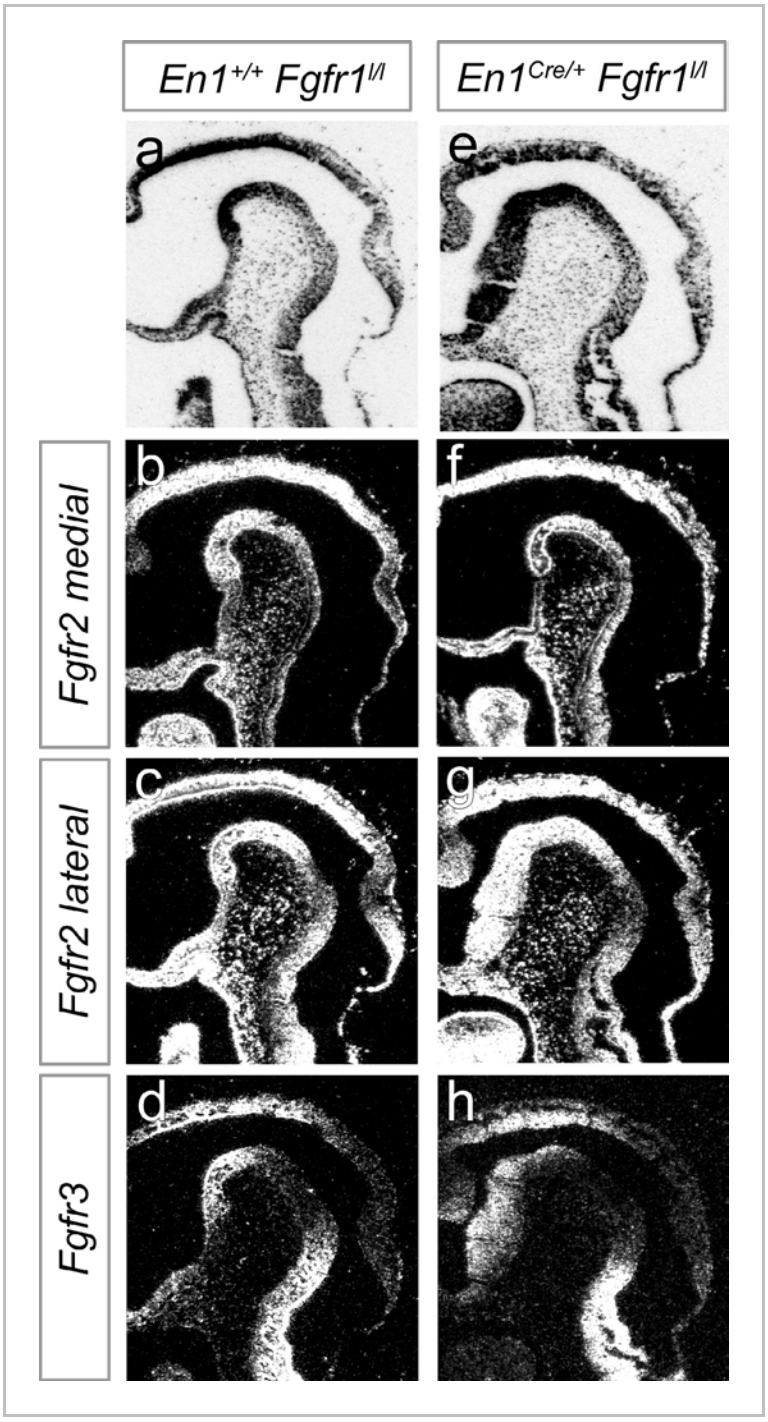

Fig. 29

Fig. 29 Similar expression of *Fgf receptors 2* and *3* in the MHR of control and *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ embryos at E10.5. Sagittal sections of control (a-d) and mutant (e-h) embryos show similar expression of *Fgfr2* (b,c,f,g) in the ventral mid-/hindbrain region and of *Fgfr3* (d,h) in the anterior midbrain and ventral hindbrain. Sections in b and f are midsagittal, in c-h parasagittal. a and e show brightfield pictures of the region.

2.3 Analysis of an allelic series of conditional *Fgfr1* and *Fgfr2* mutants

The analysis of the expression of *Fgfr1-3* in the MHR and the expression of Fgf signaling target genes in the ventral MHR of the *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ mutants suggested functional redundancy between the Fgf receptors in the MHR. Furthermore, we could show in a recent publication that loss of the *Fgfr2* alone in the MHR does not affect MHR development (Blak et al., 2007), while a conditional double knock out for *Fgfr1* and *Fgfr2* showed loss of tissue in the ventral and dorsal MHR (Blak, 2006), comparable to what had been found in the *En1*$^{Cre/+}$ *Fgf8*$^{l/l}$ mutants. This further supported the idea of redundancy of Fgfr1 and Fgfr2 in MHR development. To understand to which degree each of the two receptors contributes to the development of the MHR and to test whether there is a dependency of the MHR on the number of alleles for *Fgfr1* and *Fgfr2*, as it was shown for the engrailed genes *En1* and *En2* (Simon et al., 2001), I studied an allelic series of *Fgfr1* and *Fgfr2* compound conditional mutants.

2.3.1 Inactivation of *Fgfr2* in *En1*$^{Cre/+}$ *Fgfr2*$^{l/l}$ mice

Conventional knock out embryos for *Fgfr2* die due to an implantation defect (Arman et al., 1998). Instead, a conditional *Fgfr2* knock out line was used to generate the MHR knock out of *Fgfr2*. In *Fgfr2*$^{l/l}$ mice, exon 5 of the *Fgfr2* gene is homozygously floxed. Crossing with *En1*$^{Cre/+}$ mice resulted in excision of exon 5 after E8.5 and in functional inactivation of the gene (Blak, 2006; Blak et al., 2007). An exhaustive analysis of *En1*$^{Cre/+}$ *Fgfr2*$^{l/l}$ mutants, including Fgf signaling, showed that no phenotypic changes occurred in comparison to control littermates (Blak, 2006; Blak et al., 2007).

2.3.2 Morphological changes in an allelic series of conditional *Fgfr1* and *Ffgr2* knock out mice

The primary analysis of mutants from intercrosses of the conditional *Fgfr1* and *Fgfr2* MHR knock out mouse lines aimed at the identification of tissue loss. Therefore, morphological changes in adult and P0 mutant mice were assessed by examination of total brain anatomy (Fig. 30a,c,e,g,l,k,m,o,q) and by inspection of sagittal brain sections after Nissl staining (Fig. 30b,d,f,h,j,l,n,p,r). The genotypes of the analyzed mutants ranged from the heterozygous *Fgfr1* conditional knock out (*En1*$^{Cre/+}$ *Fgfr1*$^{l/+}$) over the single conditional knock outs (*En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ and *En1*$^{Cre/+}$ *Fgfr2*$^{l/l}$) and the compound heterozygous mutants (*En1*$^{Cre/+}$ *Fgfr1*$^{l/+}$ *Fgfr2*$^{l/l}$ and *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ *Fgfr2*$^{l/+}$) to the conditional double knock out for *Fgfr1* and *Fgfr2* (*En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ *Fgfr2*$^{l/l}$).

Conditional knock out mice heterozygous for *Fgfr1* in the MHR (*En1*$^{Cre/+}$ *Fgfr1*$^{l/+}$) were morphologically and histologically identical to the wildtype controls (Fig. 30a,b,c,d). Also *En1*$^{Cre/+}$ *Fgfr2*$^{l/l}$ mutants did not show any tissue loss in the MHR, as described elsewhere (Fig. 30e,f; Blak et al., 2007; Blak AA, 2006). Mutants with only one allele of *Fgfr1* left (*En1*$^{Cre/+}$ *Fgfr1*$^{l/+}$ *Fgfr2*$^{l/l}$) showed a variability of their phenotype, with mild (Fig. 30g,h) and severe (Fig. 30i,j) cases. This variability of the phenotype could arise from differences in the genetic background of the animals investigated. Mildly affected *En1*$^{Cre/+}$ *Fgfr1*$^{l/+}$ *Fgfr2*$^{l/l}$ mutants only had a laterally displaced IC and a malformation of folia II in the anterior cerebellar vermis (Fig. 30g,h). Severely affected *En1*$^{Cre/+}$ *Fgfr1*$^{l/+}$ *Fgfr2*$^{l/l}$ mutants had a strongly displaced IC and the cerebellar vermis was absent (Fig. 30i,j). *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ mutants showed a loss of the IC and of the cerebellar vermis but no obvious reduction in ventral tissue, as described by Trokovic et al., 2003 (Fig. 30k,l). Of the *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ *Fgfr2*$^{l/+}$ compound mutants, no surviving animals were obtained after birth. In comparison with P0 wildtype brains (Fig. 30m,n), analysis of *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ *Fgfr2*$^{l/+}$ morphology at P0 revealed a loss of the medial cerebellar anlage and of the prospective IC (Fig. 30o,p), reminiscent of the *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ phenotype (Trokovic et al., 2003). In contrast to the *En1*$^{Cre/+}$ *Fgfr1*$^{l/+}$ *Fgfr2*$^{l/l}$ mutants, the penetrance of the phenotype in the *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ *Fgfr2*$^{l/+}$ mutants was 100%, irrespective of the mixed genetic background of the breedings. A loss of ventral mid-/hindbrain tissue could not be detected by Nissl staining (Fig. 30p). Finally, *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ Fgfr2$^{l/l}$ double knock outs did not survive after birth. They lost the anlage of the SC, the IC and the cerebellum as well as tissue in the ventral MHR (see upward bend and reduced height of mesencephalic flexure; Blak AA., 2006 and Saarimäki-Vire et al., in press) (Fig. 30q,r).

These results suggest that, in the MHR, Fgfr1 and Fgfr2 are both sufficient for the development of the tissues in which they are expressed. As *Fgfr1* is expressed in all tissues

of the MHR, it can compensate for the loss of *Fgfr2*. Expression of *Fgfr2* excludes the anlage of the IC and of the cerebellar vermis and these structures are lost accordingly in the absence of *Fgfr1* expression. Furthermore, as the defects in *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ *Fgfr2*$^{l/+}$ mutants seem to be more severe than those in *En1*$^{Cre/+}$ *Fgfr1*$^{l/+}$ *Fgfr2*$^{l/l}$ mutants, Fgfr1 and Fgfr2 might be of different importance for the development of the MHR.

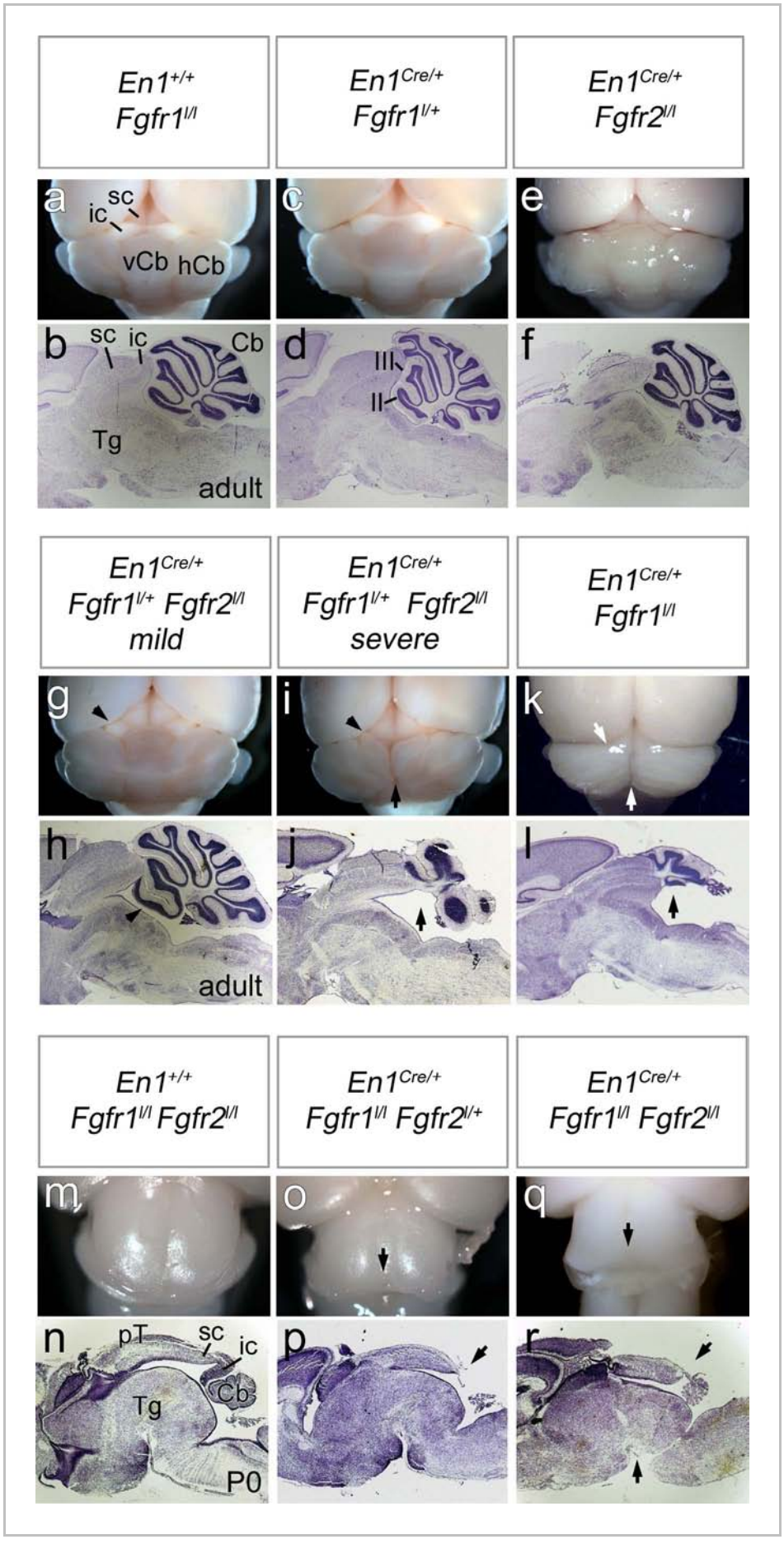
En1+/+ Fgfr1l/l
En1Cre/+ Fgfr1l/+
En1Cre/+ Fgfr2l/l
a
sc
ic
vCb
hCb
c
e
b
sc
ic
Cb
Tg
adult
d
III
II
f
En1Cre/+ Fgfr1l/+ Fgfr2l/l mild
En1Cre/+ Fgfr1l/+ Fgfr2l/l severe
En1Cre/+ Fgfr1l/l
g
i
k
h
adult
j
l
En1+/+ Fgfr1l/l Fgfr2l/l
En1Cre/+ Fgfr1l/l Fgfr2l/+
En1Cre/+ Fgfr1l/l Fgfr2l/l
m
o
q
n
pT
sc
ic
Cb
Tg
P0
p
r

Fig. 30

Fig. 30 Morphology of brains from an allelic series of conditional mutants for *Fgfr1* and/or *Fgfr2* in the MHR. Upper rows (a,c,e,g,i,k,m,o,q) show top view onto the MHR of the brains, while lower row (b,d,f,h,j,l,n,p,r) shows Nissl staining of midsagittal sections from the same brains. sc: superior colliculus, ic: inferior colliculus, vCb: cerebellar vermis, hCb: cerebellar hemisphere, II/III: foliae of cerebellum, pT: pretectum, Tg: tegmentum. Arrows point towards regions of tissue loss, arrowheads mark altered structures.

2.3.3 Analysis of the ventral MHR in *En1$^{Cre/+}$ Fgfr1$^{l/+}$* and *En1$^{Cre/+}$ Fgfr1$^{l/+}$ Fgfr2$^{l/l}$* mutant mice

The only Fgf receptor mutants in which a severe loss of tissue in the ventral MHR was observed were the *En1$^{Cre/+}$ Fgfr1$^{l/l}$ Fgfr2$^{l/l}$* double knock out mice. However, subtle changes in development or histology of the compound *Fgfr1* and *Fgfr2* conditional mutants, such as the changes observed in the ventral MHR of the *En1$^{Cre/+}$ Fgfr1$^{l/l}$* single mutants, would not have been detectable by gross morphological analysis and Nissl staining. Histological analysis of the ventral MHR in adult *En1$^{Cre/+}$ Fgfr1$^{l/+}$* and *En1$^{Cre/+}$ Fgfr1$^{l/+}$ Fgfr2$^{l/l}$* mice was carried out to detect possible alterations in these mutants.

2.3.3.1 Analysis of the ventral MHR in adult *En1$^{Cre/+}$ Fgfr1$^{l/+}$* mutants

Nissl staining and morphology of the *En1$^{Cre/+}$ Fgfr1$^{l/+}$* mutants did not reveal any changes in comparison to the control mice. To find out, whether there are changes in the histology of the ventral MHR of these mutants, immunostaining for TH, which is expressed in dopaminergic and noradrenergic neurons in the ventral midbrain (SN and VTA) and hindbrain (LC), respectively, was performed. No changes in TH expression were found in the ventral MHR of *En1$^{Cre/+}$ Fgfr1$^{l/+}$* mutant brains (Fig. 31a,b,c,d). Thus, the overall phenotype of *En1$^{Cre/+}$ Fgfr1$^{l/+}$* mice is the same as in wildtype mice, and the loss of one allele of *Fgfr1* does not interfere with correct formation of the MHR.

2.3.3.2 Analysis of the ventral MHR in adult *En1$^{Cre/+}$ Fgfr1$^{l/+}$ Fgfr2$^{l/l}$* mutants

In contrast to the *En1$^{Cre/+}$ Fgfr1$^{l/+}$* animals, adult *En1$^{Cre/+}$ Fgfr1$^{l/+}$ Fgfr2$^{l/l}$* mutants showed morphological defects in the dorsal MHR (see 2.3.2). As a consequence of the additional loss of two *Fgfr2* alleles there might also have been defects in the ventral MHR. However, immunostainings for TH in sections from adult *En1$^{Cre/+}$ Fgfr1$^{l/+}$ Fgfr2$^{l/l}$* brains did not reveal any alterations in comparison to the control littermates (Fig. 31a,b,e,f). These findings

suggest that one allele of *Fgfr1*, in the absence of *Fgfr2* expression, is still sufficient to allow for normal development of the ventral MHR.

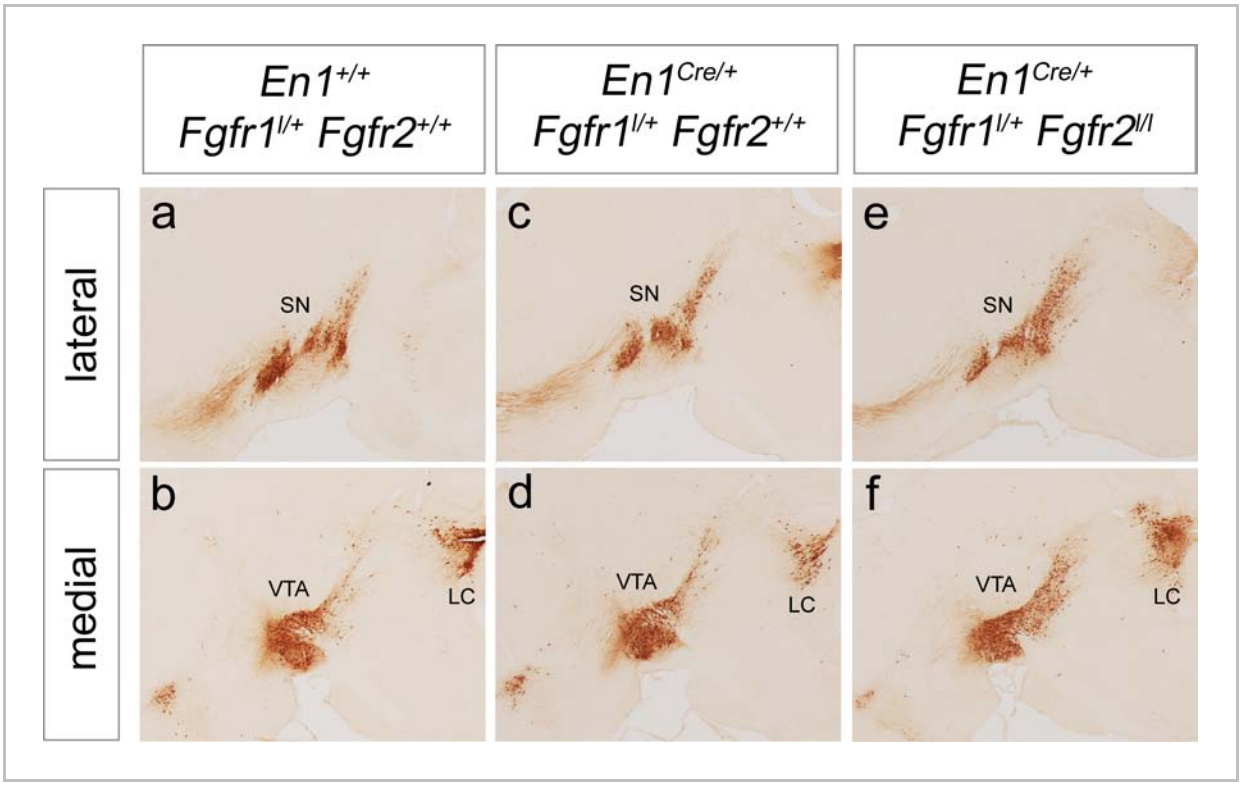

Fig. 31 No alterations in TH immunostaining in nuclei of the ventral MHR of adult *En1$^{Cre/+}$ Fgfr1$^{l/+}$* and *En1$^{Cre/+}$ Fgfr1$^{l/+}$ Fgfr2$^{l/l}$* mice. Sagittal sections from the lateral (a,c,e) and medial (b,d,f) mid-/hindbrain region of control (a,b), *En1$^{Cre/+}$ Fgfr1$^{l/+}$* (c,d) and *En1$^{Cre/+}$ Fgfr1$^{l/+}$ Fgfr2$^{l/l}$* (e,f) brains were immunostained against TH. SN: substantia nigra, VTA: ventral tegmental area, LC: locus coeruleus.

2.3.4 Analysis of the ventral MHR of *En1$^{Cre/+}$ Fgfr1$^{l/l}$ Fgfr2$^{l/+}$* mutants

En1$^{Cre/+}$ Fgfr1$^{l/l}$ Fgfr2$^{l/l}$ double knock outs have a phenotype comparable to the *En1$^{Cre/+}$ Fgf8$^{l/l}$* mutants, with complete loss of the ventral MHR (Chi et al., 2003; Blak, 2006). Furthermore, *En1$^{Cre/+}$ Fgfr1$^{l/+}$ Fgfr2$^{l/l}$* compound mutants showed dorsal phenotypes that were more severe than those of the corresponding single receptor mutants *En1$^{Cre/+}$ Fgfr1$^{l/+}$* and *En1$^{Cre/+}$ Fgfr2$^{l/l}$* (see above). To test if a gene dosage effect on patterning and specification of the ventral MHR occurs after inactivation of an additional Fgf receptor allele, the ventral MHR of compound conditional mutants in which *Fgfr1* was homozygously inactivated while one allele of *Fgfr2* was deleted in addition (*En1$^{Cre/+}$ Fgfr1$^{l/l}$ Fgfr2$^{l/+}$*) was analyzed, to be compared to the *En1$^{Cre/+}$ Fgfr1$^{l/l}$* mutants.

2.3.4.1 Expression of patterning genes is changed in the ventral MHR of *En1$^{Cre/+}$ Fgfr1$^{l/l}$ Fgfr2$^{l/+}$* embryos

Expression of the patterning genes *Otx2*, *Wnt1* and *Fgf8* was studied in E10.5 *En1$^{Cre/+}$ Fgfr1$^{l/l}$ Fgfr2$^{l/+}$* embryos, to see whether additional loss of one allele of *Fgfr2* on the homozygous *Fgfr1* conditional knock out background leads to additional alterations in patterning of the ventral MHR. In comparison to the wildtype control, *Otx2* expression was expanded caudally in the ventral MHR of *En1$^{Cre/+}$ Fgfr1$^{l/l}$ Fgfr2$^{l/+}$* mutant embryos at E10.5 and showed a disruption of the caudal expression boundary (Fig. 32a,b,g,h). The expression of *Wnt1* in the mutant embryos was also expanded caudally, when compared to the wildtype littermates and patches of *Wnt1* positive cells were found in ectopic positions in the anterior hindbrain (Fig. 32c,d,i,j). In the *En1$^{Cre/+}$ Fgfr1$^{l/l}$ Fgfr2$^{l/+}$* embryos, *Fgf8* exhibited a disruption of its anterior expression boundary and was more diffuse in expression than in the control embryo (Fig. 32e,f,k,l). Thus, the expression of the patterning genes *Otx2*, *Wnt1* and *Fgf8* in *En1$^{Cre/+}$ Fgfr1$^{l/l}$ Fgfr2$^{l/+}$* embryos resembled the changes that were observed in the *En1$^{Cre/+}$ Fgfr1$^{l/l}$* mutants (see 2.2.2.1).

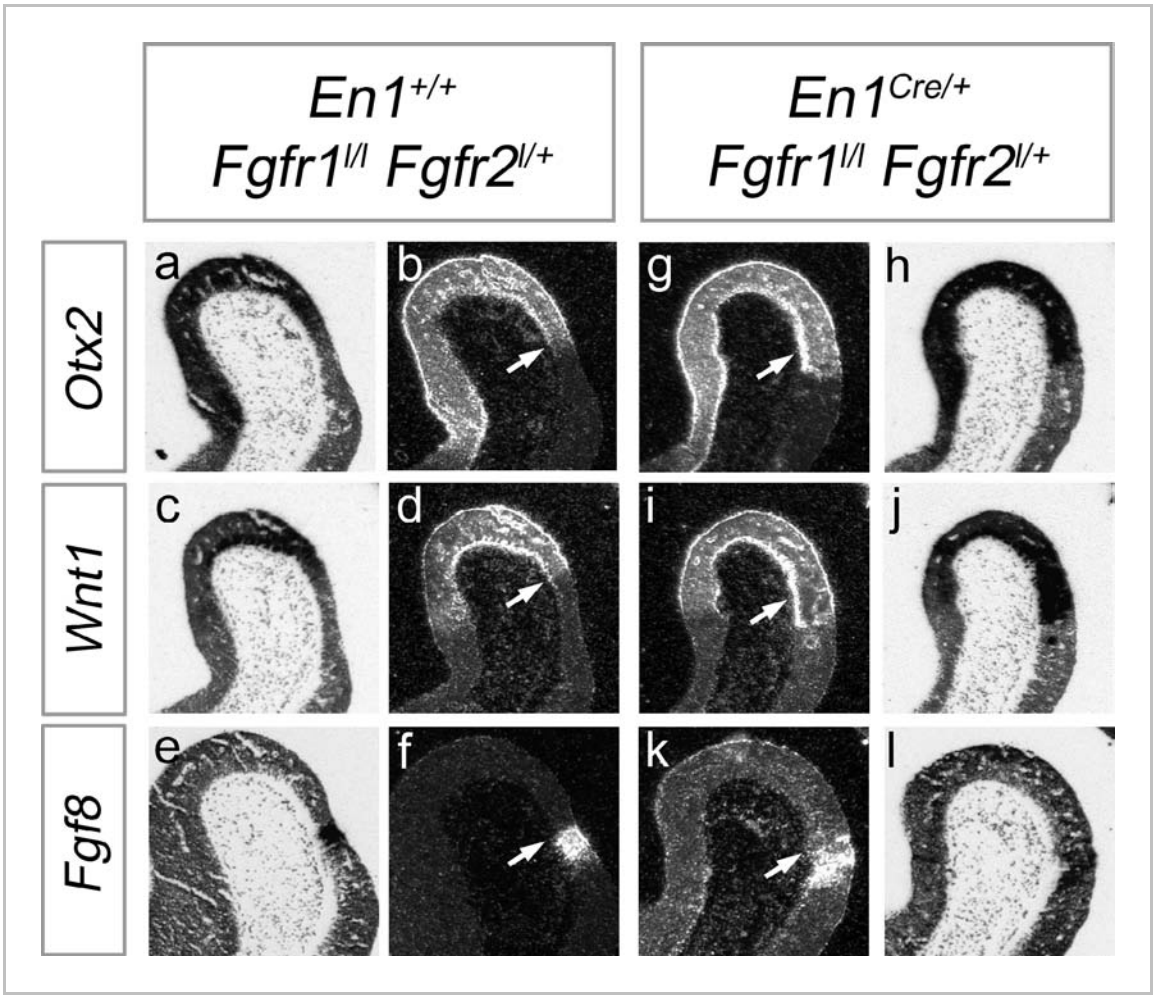

Fig. 32

Fig. 32 Expression of *Otx2* and *Wnt1* is expanded caudally and *Fgf8* expression is diffuse in the ventral MHR of *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ *Fgfr2*$^{l/+}$ embryos at E10.5. Sagittal sections of the ventral mesencephalic flexure of control (a-f) and mutant (g-l) embryos were hybridized with probes for *Otx2* (a,b,g,h), *Fgf8* (c,d,i,j) and *En1* (e,f,k,l). Brightfield sections in a,c,e,h,j and l correspond to the darkfield pictures in b,d,f,g,i and k, respectively. Arrows indicate the position that corresponds to the mid-/hindbrain boundary in the wt.

2.3.4.2 *Wnt1* expression is consistently lost in the ventral midbrain of *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ *Fgfr2*$^{l/+}$ embryos at E13.5

As in wildtype embryos, *Wnt1* expression was found in the ventral MHR of *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ mutants at E13.5. Expression of *Wnt1* was also analyzed in the MHR of *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ *Fgfr2*$^{l/+}$ embryos at E13.5, to see whether it was affected in these mutants. All of the mutant embryos analyzed showed a loss of *Wnt1* expression in the ventral MHR, while dorsal expression of *Wnt1* was the same as in the controls (Fig. 33b,d). These findings suggest a specific loss of Wnt1 in the ventral MHR of *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ *Fgfr2*$^{l/+}$ mutants at E13.5, which was not found in the *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ mutants (see 2.2.3).

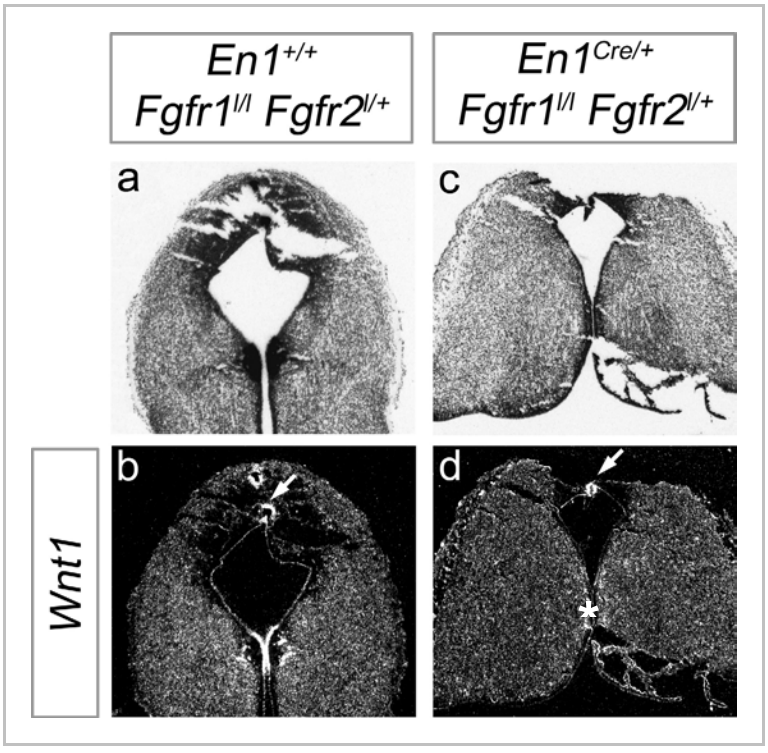

Fig. 33 *Wnt1* expression is lost in the ventral midbrain of *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ *Fgfr2*$^{l/+}$ embryos at E13.5. Coronal sections through the posterior midbrain of a control (a,b) and a mutant (c,d) embryo hybridized with radioactive probe for *Wnt1*. a and c show brightfield pictures. Arrows point to the dorsal *Wnt1* expression domain. Asterisk in d marks the region in which *Wnt1* signal is missing in the mutant embryo.

2.3.4.3 The floor plate is intact in the MHR of *En1$^{Cre/+}$ Fgfr1$^{l/l}$ Fgfr2$^{l/+}$* embryos at E13.5

Loss of *Wnt1* expression in the ventral MHR of *En1$^{Cre/+}$ Fgfr1$^{l/l}$ Fgfr2$^{l/+}$* embryos at E13.5 could have indicated a loss of ventral tissue. In-situ hybridization for the floor plate marker gene *Shh* and for *Nkx2.2*, which is expressed dorsally adjacent to *Shh*, was performed to check for the presence of ventral tissues in the MHR of the *En1$^{Cre/+}$ Fgfr1$^{l/l}$ Fgfr2$^{l/+}$* mutants. *Shh* expression was found in the mutants, indicating that the floor plate is present (Fig. 34e). Although *Nkx2.2* was also found to be expressed in the ventral MHR of *En1$^{Cre/+}$ Fgfr1$^{l/l}$ Fgfr2$^{l/+}$* embryos, the expression was diffuse and less compact than in the wildtype littermates (Fig. 34c,f). However, normal positioning of the *Nkx2.2* expression domain excluded a loss of tissue in the floor plate and basal plate of the mutant embryos.

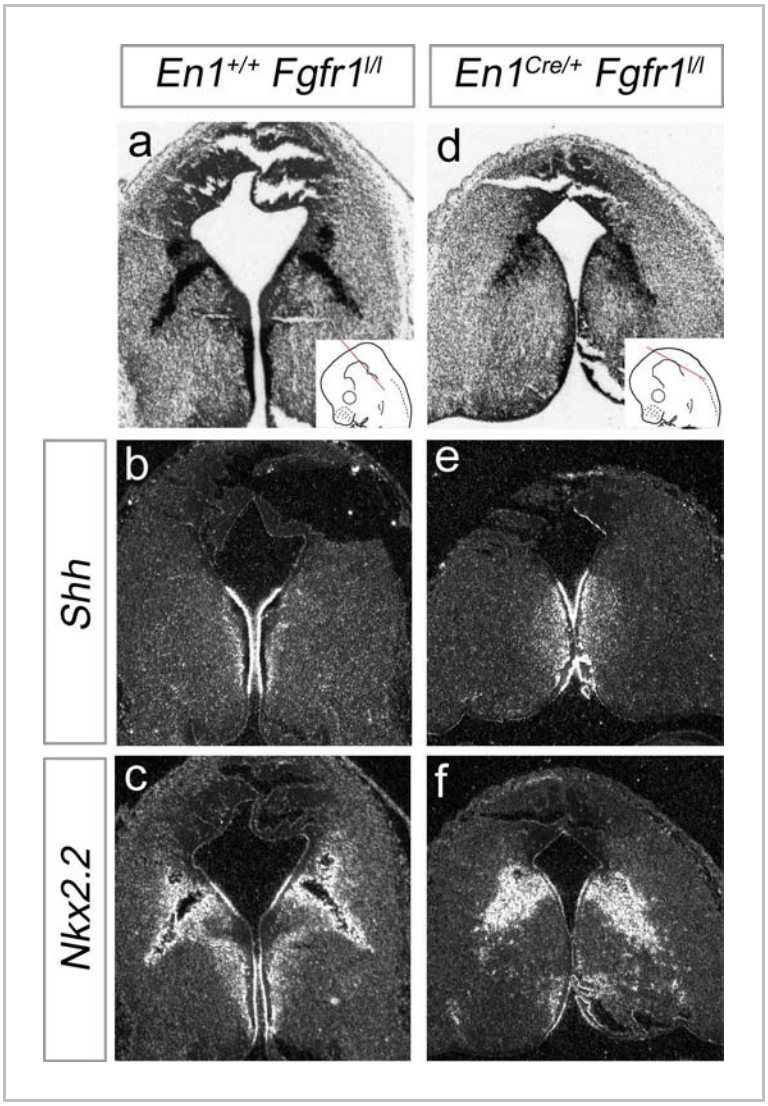

Fig. 34

Fig. 34 The floor and basal plate are intact in *En1$^{Cre/+}$ Fgfr1$^{l/l}$Fgfr2$^{l/+}$* embryos at E13.5. Coronal sections through the MHR of a control (a-c) and a mutant (d-f) embryo. *Shh* (b,e) and *Nkx2.2* (c,f) are expressed with similar d/v extent in the wt and *En1$^{Cre/+}$ Fgfr1$^{l/l}$ Fgfr2$^{l/+}$* embryo. Corresponding brightfield pictures are given in a and d. Mutants were cut further form the top, therefore the plane of sectioning is tilted towards anterior (see insets in a and d).

2.3.4.4 Caudal shift of *TH* expression and loss of anterior *Sert* expression in *En1$^{Cre/+}$ Fgfr1$^{l/l}$ Fgfr2$^{l/+}$* mutants at E13.5

Analogous to the analysis of the *En1$^{Cre/+}$ Fgfr1$^{l/l}$* mutants, the dopaminergic and serotonergic cell populations of *En1$^{Cre/+}$ Fgfr1$^{l/l}$ Fgfr2$^{l/+}$* mutant embryos in the MHR were studied at E13.5. This was important to determine whether the changes in patterning in the ventral MHR of E10.5 *En1$^{Cre/+}$ Fgfr1$^{l/l}$ Fgfr2$^{l/+}$* mutants led to alterations in the dopaminergic and serotonergic neuronal populations. Sagittal sections showed a caudal shift of *TH* expression in the ventral mesencephalic flexure of *En1$^{Cre/+}$ Fgfr1$^{l/l}$ Fgfr2$^{l/+}$* embryos as compared to the wildtype littermates (Fig. 35b,e). Concomitant with this caudal shift of *TH* expression, *Sert* expression was reduced in the anterior ventral r1 of the *En1$^{Cre/+}$ Fgfr1$^{l/l}$ Fgfr2$^{l/+}$* embryos (Fig. 35c,f). Thus, in *En1$^{Cre/+}$ Fgfr1$^{l/l}$ Fgfr2$^{l/+}$* mutants, the anterior serotonergic neurons were lost at E13.5, similar to the *En1$^{Cre/+}$ Fgfr1$^{l/l}$* mutants. However, while the *TH* expression domain in the *En1$^{Cre/+}$ Fgfr1$^{l/l}$* embryos was expanded caudally, *TH* expression was not expanded but rather shifted caudally in the *En1$^{Cre/+}$ Fgfr1$^{l/l}$ Fgfr2$^{l/+}$* embryos (compare 2.2.5.2).

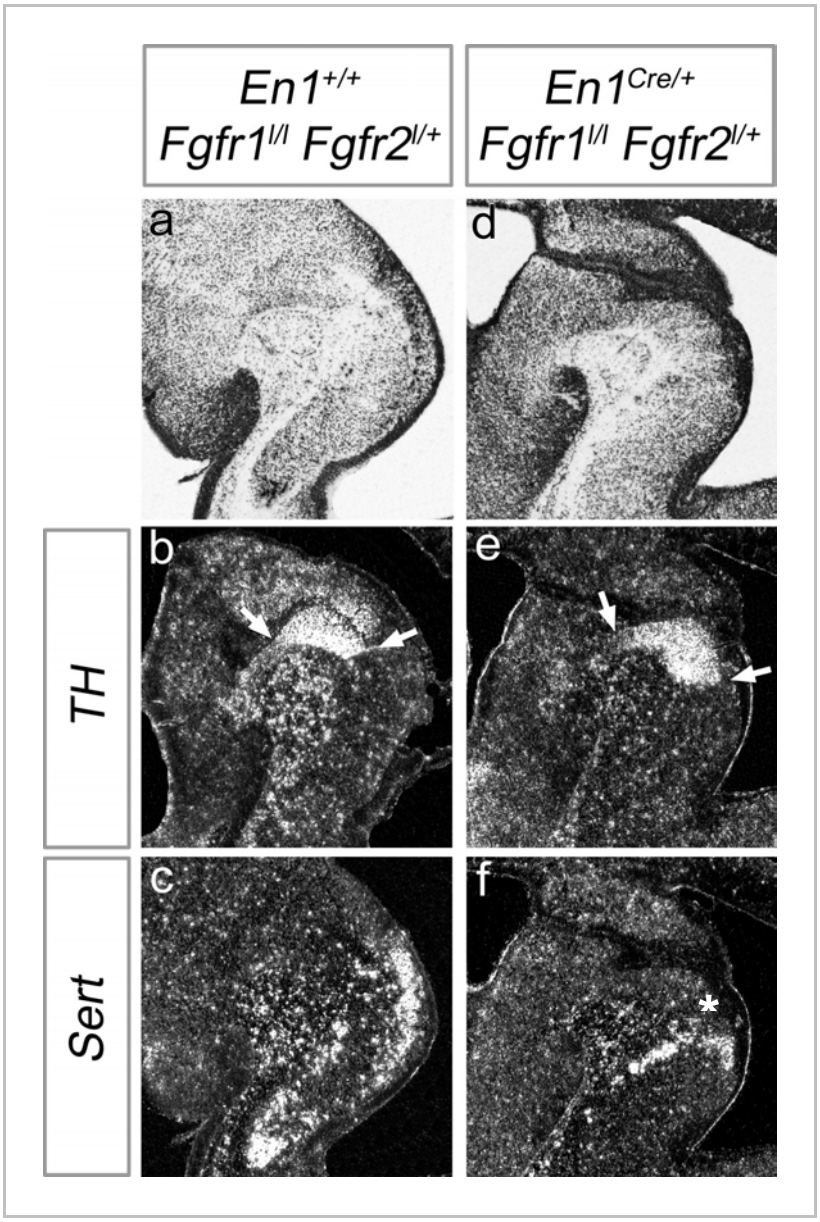

Fig. 35 Dopaminergic neurons are shifted caudally and serotonergic neurons are reduced in the ventral MHR of *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ *Fgfr2*$^{l/+}$ embryos at E13.5. Sagittal sections of the ventral mesencephalic flexure of wt (a-c) and mutant (d-f) embryos hybridized with probes for *TH* (b,e) and *Sert* (c,f). Brightfield pictures are given in a and d. Asterisk in f marks absence of signal for *Sert* in the anterior r1. Arrows demarcate the boundaries of TH expression in the ventral MHR.

2.3.4.5 Changes in dopaminergic and serotonergic neurons are persistent and specific in *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ *Fgfr2*$^{l/+}$ mutants

The integrity of neuronal populations in the MHR of *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ *Fgfr2*$^{l/+}$ mutants was checked at P0 to test for the specificity and persistence of the alterations observed in the dopaminergic and serotonergic system at earlier stages. *In-situ* hybridization was performed for the dopaminergic markers *TH* and *Dat*, for the serotonergic marker *Sert* and for the cholinergic marker *VAChT*.

Comparable to the findings in the *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ mutants, expression of *TH* and *Dat* was absent from the midline of the ventral midbrain at this stage (Fig. 36e,f).

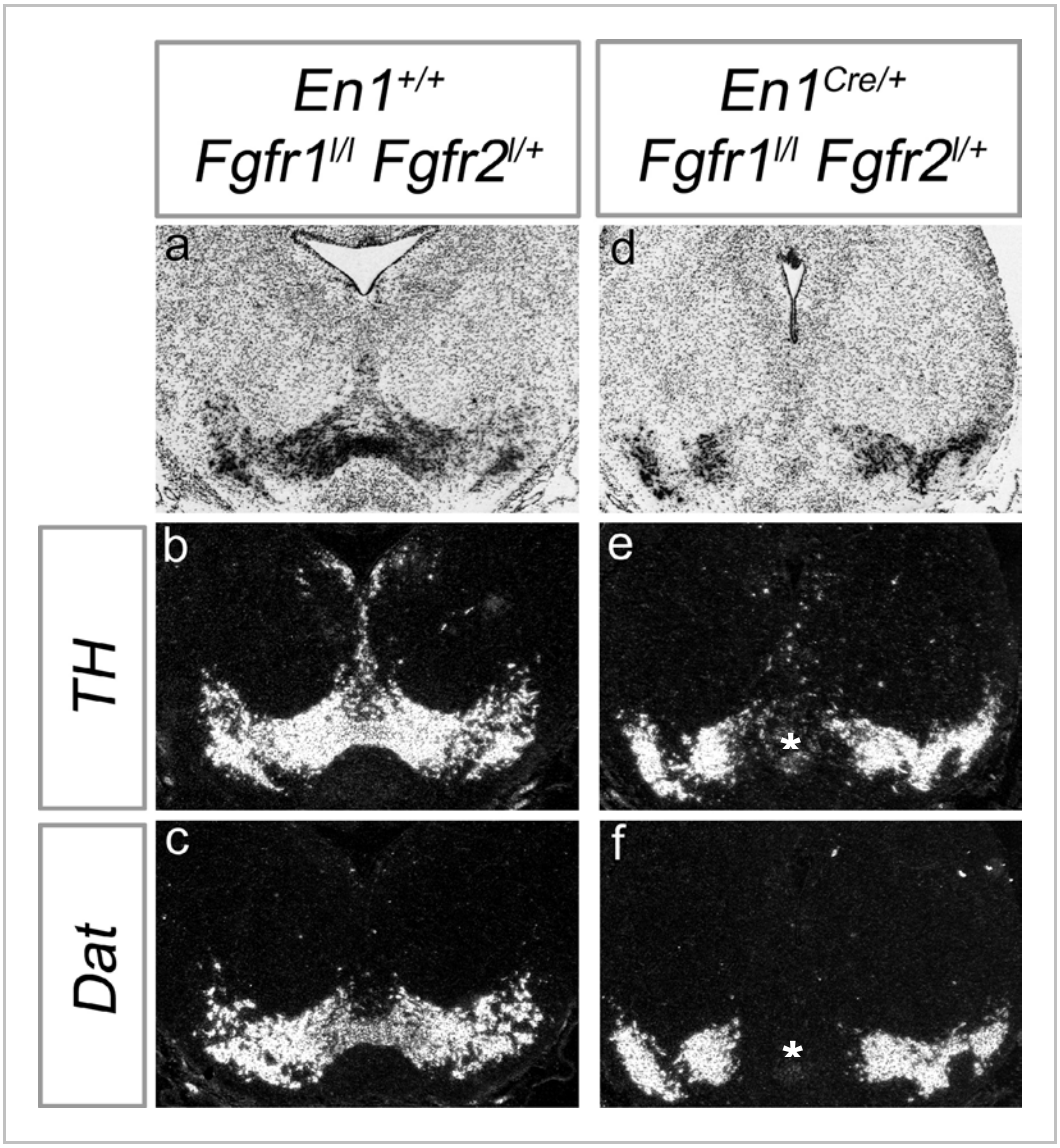

Fig. 36 Dopaminergic neurons are absent from the medial VTA in the ventral MHR of P0 brains from *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ *Fgfr2*$^{l/+}$ mice. Coronal sections from the MHR of control (a-c) and *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ *Fgfr2*$^{l/+}$ (d-f) animals hybridized with radioactive probes for *TH* (b,e) and *Dat* (c,f). Brightfield pictures are given in a and e. Asterisks mark the VTA of mutant mice, from which signal is absent.

Sert expression in the DR nucleus was also reduced in *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ *Fgfr2*$^{l/+}$ mutants at P0 (Fig. 37b,e), similar to the phenotype found in the *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ mutants. The expression of *VAChT* in the nuclei of the 3rd and 4th cranial nerve was the same in *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ *Fgfr2*$^{l/+}$ mutants and control animals (Fig. 37c,f). Also this was similar to what was found in the *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ mutants and suggested that the observed changes in the dopaminergic and serotonergic neurons are specific to these two neuronal populations (compare 2.2.7.6).

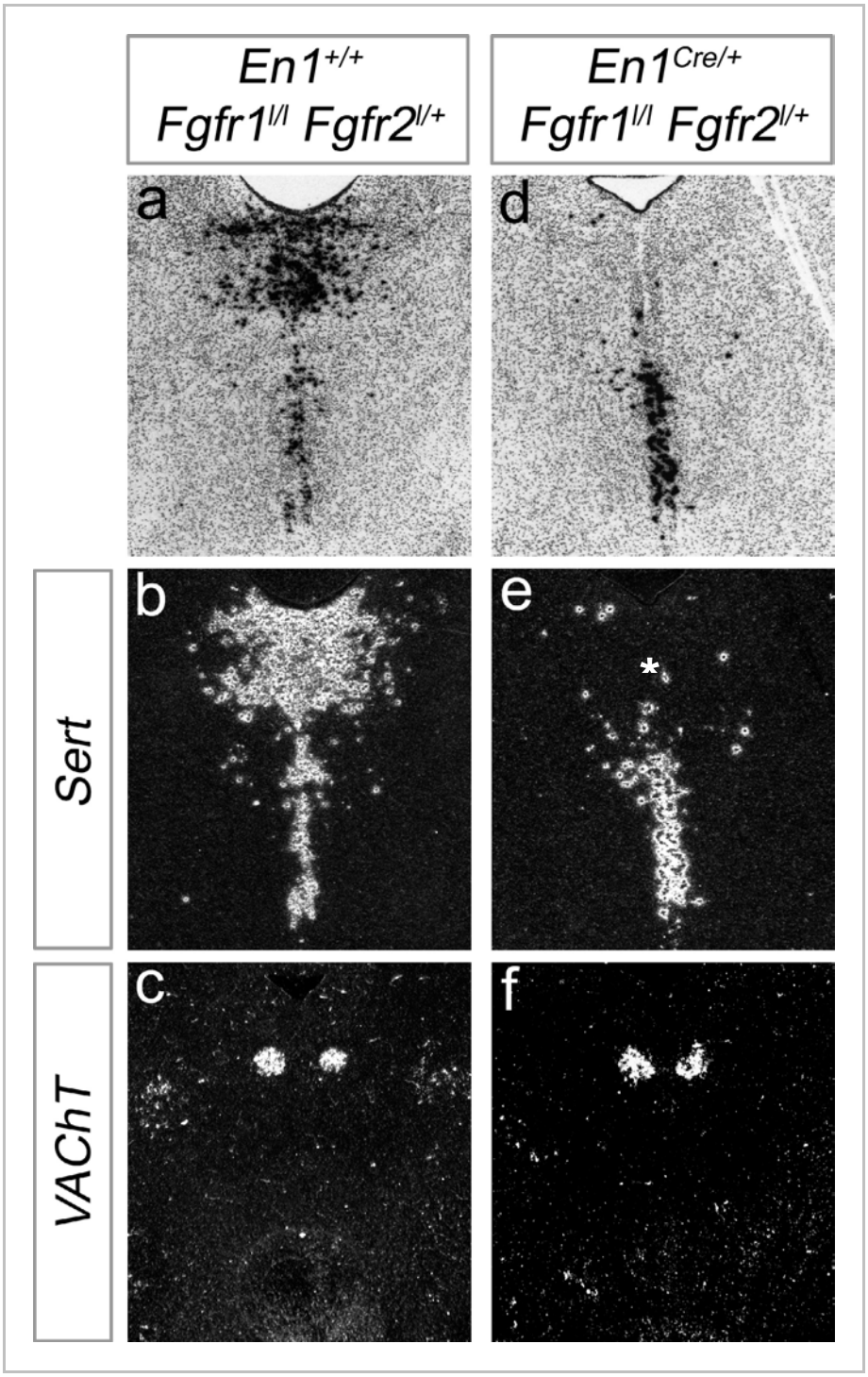

Fig. 37 Serotonergic neurons are reduced whereas cholinergic neurons are normal in *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ *Fgfr2*$^{l/+}$ mice at P0. Coronal sections from the MHR of control (a-c) and *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ *Fgfr2*$^{l/+}$ mutant (d-f) mice were hybridized with probes for *Sert* (b,e) and *VAChT* (c,f). Brightfiled pictures of the region are given in a and e. Asterisk in e marks the DR, from which *Sert* expression is absent in the mutant.

2.3.4.6 Fgf signaling is active in the ventral MHR of *En1$^{Cre/+}$ Fgfr1$^{l/l}$ Fgfr2$^{l/+}$* mutants at E10.5

As in the *En1$^{Cre/+}$ Fgfr1$^{l/l}$* mutants, I analyzed the expression of the Fgf signaling target gene *Sef1* in the MHR of *En1$^{Cre/+}$ Fgfr1$^{l/l}$ Fgfr2$^{l/+}$* mutants at E10.5.
At this stage, *Sef1* expression was detected in the ventral MHR of the mutant embryos, indicating that Fgf signaling was active despite the loss of both alleles for *Fgfr1* and one allele for *Fgfr2* (Fig. 38d). This finding mirrored what was seen in the *En1$^{Cre/+}$ Fgfr1$^{l/l}$* mutants (2.2.8.1). However, a downregulation of *Sef1* at later stages cannot be ruled out.

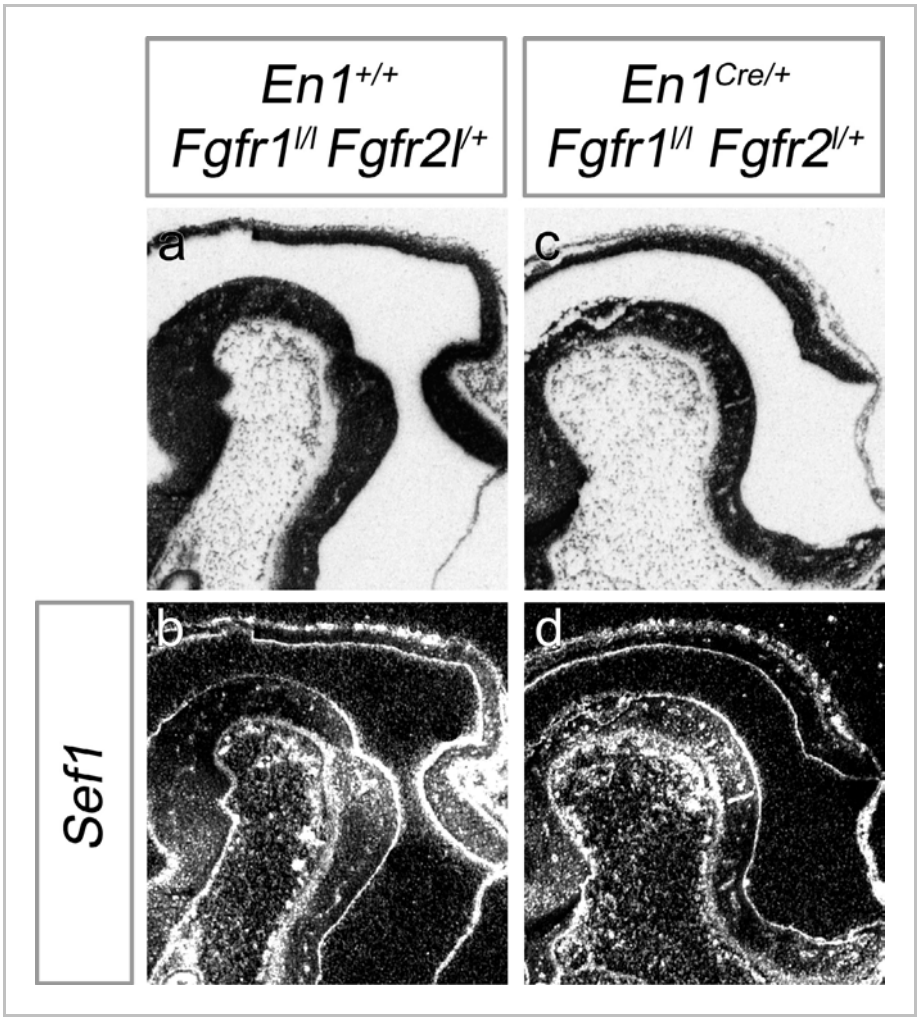

Fig. 38 The Fgf signaling target *Sef1* is expressed in the ventral MHR of *En1$^{Cre/+}$ Fgfr1$^{l/l}$ Fgfr2$^{l/+}$* embryos at E10.5. Sagittal sections of the MHR of a control (a,b) and a mutant (c,d) embryo hybridized with probe for *Sef1* (b,d). Corresponding brightfield pictures are given in a and c.

Taken together, the additional loss of one allele of *Fgfr2* on an *Fgfr1* homozygous background in the MHR led to an additional defect in the maintenance of *Wnt1* expression. Early *TH* expression in the *En1$^{Cre/+}$ Fgfr1$^{l/l}$ Fgfr2$^{l/+}$* embryos did not precisely resemble the

expansion that was seen in the *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ mutants, but the absence of dopaminergic neurons from the VTA was similar in both mutants at P0. Furthermore, a reduction in the serotonergic neurons of the anterior DR was found in both mutants. Therefore, from the preliminary results obtained so far, the *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ *Fgfr2*$^{l/+}$ mutants were very similar to the *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ single knock outs, with only a specific additional loss of *Wnt1* expression at E13.5. To find out whether other additional defects are present in the ventral MHR of the *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ *Fgfr2*$^{l/+}$ mutants, a more exhaustive analysis would need to be carried out.

3 Discussion

3.1 Abstract

In the work presented here, I reveal distinct and common functions of Fibroblast growth factor receptors in the development of the midbrain and hindbrain. Conditional knock out mice for *Fgfr1*, in which *Fgfr1* is inactivated in the midbrain and anterior hindbrain, lose tissue in the dorsal but not in the ventral MHR. This allowed me to do an analysis of the ventral MHR in mice in which *Fgfr1* was inactivated in that area. The analysis showed that the Fgfr1 plays a specific role in the separation of midbrain and hindbrain patterning factors and in the establishment and positioning of dopaminergic and serotonergic neurons that arise close to the midbrain-/hindbrain boundary. The role of Fgfr1 and Fgfr2 in development of tissue in the MHR became clear from the study of an allelic series of conditional knock out mouse lines for these two receptors. The phenotype of the resulting mutant mice suggests that the expression pattern of the Fgf receptors determines their contribution to the formation of tissue in the midbrain and anterior hindbrain. Furthermore, some functions of the Fgf receptors in the MHR depend on the total number of alleles of Fgf receptors that are expressed.

3.2 A specific role for Fgfr1 in development of the ventral MHR

Although Fgf signaling in the ventral MHR was found to be active, the analysis of the $En1^{Cre/+}$ $Fgfr1^{l/l}$ mutants showed defects in the segregation of patterning factors from the ventral midbrain and hindbrain. Dopaminergic neurons of the midbrain were less densely packed in the embryo and extended into the anterior hindbrain, where serotonergic neurons were reduced. In the adult mutants the total number of dopaminergic neurons was unchanged. However, mDA neurons were absent from the medial VTA but instead ectopic DA neurons were found in the anterior lateral hindbrain in $En1^{Cre/+}$ $Fgfr1^{l/l}$ animals. Expression of Calbindin and Girk2 in these ectopic neurons suggests that they have midbrain identity. The number of 5-HT neurons in the DR nucleus of the adult mutant anterior hindbrain was reduced by 45%. No reduction was observed in the size of the nucleus of the 4^{th} nerve, which is located in close vicinity to the DR nucleus and this result implies that the observed changes are specific for the mDA and 5-HT neurons at the mid-/hindbrain boundary.

3.2.1 Fgfr1 is necessary for the maintenance of the compartment boundary between the midbrain and the hindbrain

The genes *Otx2*, *Emx2* and *Wnt1,* which are expressed in the midbrain, were found to be ectopically expressed in the anterior hindbrain of the *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ mutant embryos. On the other hand, the hindbrain expression domain of *Nkx2.2* was shifted caudally in the same area. Furthermore, *Otx2*, *Emx2*, *Wnt1* and *Nkx2.2* displayed a diffuse expression boundary and patches of cells expressing these genes were found at ectopic positions. Expression of *Fgf8* was less compact and extended slightly into the caudal midbrain, but was found at a comparable position in the hindbrain of wildtype and mutant embryos. The diffuse expression boundaries and ectopic patches of cells expressing the patterning genes *Otx2*, *Emx2*, *Nkx2.2*, *Wnt1* and *Fgf8* suggest a loss of the strict compartment boundary between the midbrain and the hindbrain that is normally established around E9, the time at which *Fgfr1* is fully inactivated in the MHR of *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ mutants (Trokovic et al., 2003; Zervas et al., 2004). In the first description of the *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ mutants a downregulation of the adhesion molecule *PB-Cadherin* was found and it was speculated that this impairs the cell sorting mechanisms which are normally responsible for the separation of cells from the midbrain and hindbrain (Trokovic et al., 2003). Reduced cell adhesion and a defect in cell recognition might allow for the migration of cells from one compartment into the other and thereby lead to ectopic patches of cells expressing midbrain patterning molecules in the anterior hindbrain and to diffuse expression boundaries at the interface between the mid- and hindbrain, as observed in the *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ mutant embryos. In their report, Trokovic et al. also showed diffuse expression boundaries of *Otx2, Gbx2* and *Wnt1* in the dorsal MHR of E9.5 *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ mutant embryos (Trokovic et al., 2003). My findings show that expression boundaries at the mid-/hindbrain interface are diffuse also in the ventral MHR of *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ embryos and expand the number of genes that have been shown to display diffuse expression boundaries.

3.2.2 Fgfr1 is necessary for correct patterning of the ventral MHR

Ectopic patches of cells expressing *Otx2* and *Wnt1* were found in the anterior r1 of the *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ embryos at E10.5. Furthermore, *Gbx2* expression in the anterior hindbrain was downregulated in the mutants (data not shown). The fact that *Gbx2* expression in r1 is lost in the *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ mutants and that ectopic *Otx2* and *Wnt1* were not repressed in the anterior hindbrain, as it is the case in normal MHR development (Millet et al., 1999; Prakash and Wurst, 2004; Sato et al., 2004), is probably a consequence of reduced *Fgf8* signaling by Fgfr1 early in the MHR. Although at E10.5 target genes of Fgf signaling are expressed

throughout the ventral MHR, Fgf signaling might be impaired at earlier time points in parts of the ventral MHR. Our analysis of the expression of *Fgfr1-3* in the MHR of wildtype mouse embryos showed, that the *Fgf8* positive tissue in the most anterior hindbrain expresses *Fgfr1*, but is free of *Fgfr2* and *Fgfr3* expression before E9.5 (Blak et al., 2005). The inactivation of *Fgfr1* in the MHR of the $En1^{Cre/+}$ $Fgfr1^{l/l}$ mutants begins as early as E8.5 (Trokovic et al., 2003). Therefore, in $En1^{Cre/+}$ $Fgfr1^{l/l}$ mutant embryos, there might exist a short period of time between E8.5 and E9.5 in which no Fgf receptor is expressed in the *Fgf8* positive tissue of the most anterior hindbrain and Fgfr mediated signaling is not effective in that region. Downregulation of *Gbx2* expression in the anterior hindbrain of the mutants might occur during this time interval. Indeed, it has been shown before that the hindbrain patterning gene *Gbx2* can be induced by FGF8 coated beads in ectopic positions and that *Gbx2* expression is not maintained in conditional *Fgf8* knock out mice (Liu et al., 1999; Chi et al., 2003). Ectopic application of FGF8 also leads to an inhibition of *Otx2* expression (Liu and Joyner, 2001b). Thus, a loss of Fgf8 signaling by Fgfr1 between stages E8.5 and E9.5 could allow for caudal expansion of *Otx2* expression into the most anterior r1. In the $En1^{Cre/+}$ $Fgfr1^{l/l}$ mutants, signal transduction by Fgfr1 is lost and as a consequence *Gbx2* expression is not maintained and *Otx2* expression is expanded into the anterior hindbrain, consistent with the role of *Fgf8* in the regulation of patterning in the MHR.

It is further interesting that there exists an obvious difference in the patchy expression boundaries of the genes *Otx2*, *Emx2*, *Wnt1* and *Nkx2.2* in r1 of the $En1^{Cre/+}$ $Fgfr1^{l/l}$ embryos and the sharp caudal shift of the expression boundaries of the dopaminergic marker gene *TH* and the serotonergic marker genes *Pet1* and *Sert*. The patchy distribution of cells expressing *Otx2*, *Emx2*, *Wnt1* and *Nkx2.2* in the anterior hindbrain of the $En1^{Cre/+}$ $Fgfr1^{l/l}$ mutants implies that the regulation of their expression is cell autonomous and that their patchy expression boundaries probably arise through erroneous cell migration. The most caudal patch of cells expressing *Otx2* coincides with the sharp caudal boundary of *TH* expression and is found just anterior to the sharp expression boundary of *Pet1* and *Sert* in the mutants. This coincidence of patchy and sharp expression boundaries hints towards a non-cell autonomous mechanism, maybe dependent on the expression of *Otx2* and *Wnt1*, for the regulation of the expression of *TH*, *Pet1* and *Sert*. This would be in accordance with observations made in mutants in which the expression of *Otx2* and *Gbx2* was shifted in the MHR (Broccoli et al., 1999; Brodski et al., 2003). In such mutants, the boundary between the expression domains of dopaminergic and serotonergic marker genes was shifted to the same position as the *Otx2*/*Gbx2* interface.

3.2.3 Midbrain dopaminergic and hindbrain serotonergic neurons are specifically affected in *En1$^{Cre/+}$ Fgfr1$^{l/l}$* mice

Besides the changes observed in patterning, dopaminergic and serotonergic neurons were also affected in the *En1$^{Cre/+}$ Fgfr1$^{l/l}$* mutant mice. The mDA neuron domain was expanded caudally in the mutant embryos. In adult mutant animals mDA neurons were found at ectopic positions in the anterior hindbrain, whereas no dopaminergic neurons were found in the VTA. Opposite to the expansion of dopaminergic neurons in the embryos, 5-HT neurons were reduced in the anterior hindbrain of the *En1$^{Cre/+}$ Fgfr1$^{l/l}$* embryos. This reduction in the number of 5-HT neurons in the anterior r1 persisted into adulthood. Although in the *En1$^{Cre/+}$ Fgfr1$^{l/l}$* mutants the neurons of the 3rd and 4th cranial nerve and of the LC were not as densely packed as in the wildtype animals, they were in the correct position and no reduction in the size of these nuclei was observed.

Ectopic positioning of dopaminergic neurons in the anterior hindbrain could be explained by erroneous migration of mDA neurons, or by ectopic induction of dopaminergic neurons in this region. Dopaminergic neurons from the caudal midbrain could have migrated into the anterior hindbrain in the mutant mice, which would also correlate well to the caudal expansion of the mDA neuron domain in the *En1$^{Cre/+}$ Fgfr1$^{l/l}$* embryos. Such an ectopic migration of mDA neurons might be a consequence of the loss of the compartment boundary between the midbrain and the hindbrain. Altered cell sorting and migration would also explain that Calbindin and Girk2 expressing mDA neurons are found at ectopic positions in the anterior hindbrain of the adult mutants and are absent from the medial VTA in the midbrain and that the total number of dopaminergic neurons in the MHR is not changed. This hypothesis would be supported by a publication in which SN and VTA dopaminergic neurons have been shown to depend differentially on *Pitx3* (Maxwell et al., 2005). In this publication, the DA neurons of the VTA have been suggested to originate from the caudal part of the midbrain which is located close to the *Fgf8* expression at the mid-/hindbrain boundary in mouse embryos, whereas the DA neurons of the SN probably derive from the more anterior midbrain. In the adult *En1$^{Cre/+}$ Fgfr1$^{l/l}$* mutant animals, the medial VTA does not contain dopaminergic neurons, while the SN is not affected. Thus, the VTA DA neurons that develop close to the mid-/hindbrain boundary might have migrated into the anterior hindbrain, while the SN DA neurons which develop further away from the boundary are normal. However, the ectopic mDA neurons express both, Calbindin and Girk2, and have therefore not a definitive medial VTA phenotype, which argues against a simple migration of mDA neurons from the medialVTA into the anterior hindbrain. Furthermore, specific markers for two major neuronal populations of the CNS - glutamatergic and GABAergic neurons - have not been studied in the *En1$^{Cre/+}$ Fgfr1$^{l/l}$* mutants. It might therefore be possible, that the neurons of the medial

VTA become transformed into glutamatergic or GABAergic neurons in the absence of *Fgfr1* and that ectopic mDA neurons become induced *de novo* in the anterior hindbrain. However, analysis of the expression of *Pou4f1*, *VAChT* and *DBH*, which are characteristic for neurons of the red nucleus, the cranial nerve nuclei and the noradrenergic neurons of the locus coeruleus respectively, in the MHR of *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ mutant mice, did not reveal the induction of another neuronal fate in neurons of the VTA (data not shown). Although no obvious loss of Nissl stained cells was visible in the VTA of mutant animals, a third possibility to explain the loss of DA neurons in this area would be that these neurons die. A detailed study on the expression of glutamatergic and GABAergic marker genes and on cell death in the ventral MHR of the mutant mice might help to understand the fate of the VTA DA neurons in the *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ mice.

The reduction of 5-HT neurons in the anterior hindbrain of *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ mutant mice seems to be a consequence of changes in differentiation of the neurons. Differentiation of serotonergic neurons in the anterior hindbrain depends on the sequential action of the transcription factors Mash1 and Pet1 (Pattyn et al., 2004). Other transcription factors are likely to be necessary for serotonergic specification and differentiation in r1 in the mouse, but have not been described so far. Expression of *Mash1*, a bHLH transcription factor that had been shown to be necessary for the development of all serotonergic neurons in the hindbrain (Pattyn et al., 2004), was found in the ventral hindbrain of *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ embryos at E13.5, reflecting the wildtype expression pattern (data not shown). However, Jukkola and colleagues could show that *Mash1* is downregulated in the anterior ventral r1 of *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ mice at E10.5, the stage when serotonergic differentiation begins (Jukkola et al., 2006c). The ETS transcription factor *Pet1* has also been implicated in the differentiation of 5-HT neurons (Hendricks et al., 2003). Expression of *Pet1* was lost in the anterior hindbrain of three of four mutant embryos at stage E13.5, whereas one mutant embryo only showed reduced expression in the same region, suggesting a defect in the maintenance of *Pet1* expression in the *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ mutant embryos. Jukkola et al. also reported downregulation of *Pet1* expression in the *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ mice as early as E11.5 (Jukkola et al., 2006b). All mutant embryos analyzed showed reduced expression of *Sert* at E13.5. Besides *Mash1* and *Pet1*, *Nkx2.2* is known to be necessary for the correct development of serotonergic neurons in rhombomeres 2-7 (Pattyn et al., 2003). Its expression is also upregulated in the ventral midbrain of *En1*$^{Cre/+}$ *Otx2*$^{l/l}$ mutant mice which show ectopic induction of 5-HT neurons in that region (Puelles et al., 2004). However, *Nkx2.2* knock out mice did not show a loss of 5-HT neurons in rhombomere 1, making it unlikely that *Nkx2.2* alone is essential for serotonergic neuron development in r1 (Briscoe et al., 1999; Ding et al., 2003). An ortholog of *Nkx2.2* is *Nkx2.9* (Pabst et al., 1998), which has been suggested to be functionally redundant to Nkx2.2 (Pabst et al., 2003). Analysis of the expression of *Nkx2.9* in the MHR of wildtype and

En1$^{Cre/+}$ *Fgfr1*$^{l/l}$ mutant embryos showed no clear correlation in the expression of this gene with the expression of the serotonergic marker gene *Sert* at E13.5 (data not shown). However, expression of *Nkx2.9* might overlap with that of *Nkx2.2* and 5-HT marker genes at earlier stages and both factors might have redundant functions in the specification of 5-HT precursor cells. This would also explain that no loss of 5-HT neurons in r1 was observed in the *Nkx2.2*$^{-/-}$ mice (Briscoe et al., 1999; Ding et al., 2003). Therefore, functional redundancy between *Nkx2.2* and *Nkx2.9* might be an explanation for the independence of 5-HT neurons in anterior r1 from *Nkx2.2*. Further studies on the coexpression of *Nkx2.2*, *Nkx2.9* and *Pet1* at earlier developmental stages in wildtype and *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ mice are planned, but could not be finished for this dissertation. Nevertheless, the loss of *Mash1* and *Pet1* expression close to the *Fgf8* signal at the mid-/hindbrain boundary most likely contributes to the absence of *Sert* expression from anterior r1 in the *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ mutant mice.

It is also interesting to note that not all 5-HT neurons in r1 are lost after inactivation of *Fgfr1* in the MHR. The remaining 5-HT neurons in embryonic r1 of *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ mutants are located within the region in which *Fgfr1* is inactivated but do still develop a normal serotonergic phenotype that is persistent into adulthood. A subdivision of the 5-HT neuron population in r1 is therefore likely, with one anterior subgroup close to the mid-/hindbrain boundary depending on signaling by Fgfr1 and another group of 5-HT neurons in caudal r1 which does not depend on Fgfr1 mediated signaling. This subdivision of the 5-HT neurons is comparable to the separate subpopulations of dopaminergic neurons in the midbrain. A differential dependence of anterior and posterior r1 on Fgf signaling was also observed in zebrafish mutants for *Fgf8*, in which *Otx2* expression is expanded into the anterior r1, while the caudal r1 develops normal in the absence of wildtype *Fgf8* (Jaszai et al., 2003). How the development of caudal 5-HT neurons in r1 is regulated in detail is not clear so far and will have to be analyzed in a separate project. My studies on the development of the anterior 5-HT neurons in r1 suggest that these depend on the induction and maintenance of *Pet1* expression by signaling through Fgfr1 and that *Sert* expression is not initiated in these cells as a result of the absence of *Mash1* and *Pet1*. Whether the cells that would become 5-HT neurons in the anterior ventral r1 adopt another fate or are lost due to cell death is not yet clear. A more detailed analysis of neuronal cell type specific marker genes in the ventral MHR and visualization of cell death in this region could help to understand the fate of these cells.

Other neuronal populations in the ventral MHR, such as the neurons of the nuclei of the 3rd and 4th nerve, did not show changes comparable to those of the dopaminergic and serotonergic neuronal populations. In addition, the expression of *Pou4f1*, which marks the glutamatergic neurons of the red nucleus, was not changed in the *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ mutants (data not shown). Although the neurons of the LC were less densely packed and the

distinction between the 3rd and 4th nerve nuclei was less clear in the *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ mutants, none of these cell populations showed ectopically positioned neurons or an obvious qualitative increase or reduction in the number of neurons. Therefore, the dopaminergic and serotonergic neurons in the MHR of *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ mutants are specifically affected after loss of *Fgfr1*. Reasons for the restriction of severe changes to the mDA and 5-HT neurons in the MHR might be twofold. First, although the noradrenergic neurons of the LC have been shown to depend on Fgf8 (Guo et al., 1999; Lam et al., 2003), their origin is in a more caudal and dorsal position than that of the 5-HT neurons (Aroca et al., 2006). Similarly, the neurons of the red nucleus in the midbrain are born in a more anterior position (Agarwala and Ragsdale, 2002). In these regions, *Fgfr2* is expressed from E8.5 on and might rescue development of the neurons in the LC and red nucleus. Second, the cholinergic neurons of the 3rd and 4th nerve nuclei have been proposed to be specified independently of Fgf signaling (von Bartheld and Bothwell, 1992). Thus, the mDA neurons of the VTA and the anterior 5-HT neurons might be the only neuronal populations altered in the *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ mutants, because they are the only neurons whose Fgf8 responsive precursor cells are located close to the mid-/hindbrain boundary between E8.5 and E9.5.

3.2.4 Fgf signaling in the MHR of *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ embryos

Fgf signaling in the ventral MHR of the mutants was found to be at least partially intact. The genes *Sef1* and *Spr1* are targets of the Ras/MAPK (Minowada et al., 1999; Tsang et al., 2002) signaling pathway, while *MKP3* expression has been described to be regulated via the PI3K/Akt (Echevarria et al., 2005) pathway. Although their expression is absent in the dorsal MHR, all three of these genes were expressed in the ventral MHR of the *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ mutant embryos at E10.5, suggesting that the PI3K/Akt and Ras/MAPK intracellular signal transduction pathways are activated by Fgf signaling at this stage. Expression of *Spry1* and *MKP3* has also been described before in the ventral MHR of *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ embryos after E9.5 (Jukkola et al., 2006a). However, in early E9.5 mutant embryos it was shown that the expression of *Spry1* and the two effector genes of Fgf signaling *Pea3* and *Erm* (Raible and Brand, 2001; Roehl and Nusslein-Volhard, 2001) was initially downregulated in a stripe of tissue at the mid-/hindbrain boundary (Trokovic et al., 2003). The stripe of tissue in which *Spry1*, *Pea3* and *Erm* were downregulated in the *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ mutants at this early stage corresponded well to the expression domain of *Fgf8*. *Fgfr2* and *Fgfr3* are candidates to mediate the activation of the PI3K/Akt and Ras/MAPK intracellular signal transduction pathways in the ventral MHR of the *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ mutants, but between E8.5 and E9.5 their expression is restricted to the tissue surrounding the *Fgf8* expression domain. Therefore, the specific defects which arise in the ventral MHR of the mutant animals, i.e. the migration of

cells from the midbrain into the hindbrain and the effects seen on the VTA and DR neurons, might be a consequence of the absence of Fgf signaling at the mid-/hindbrain boundary between E8.5 and E9.5 after inactivation of *Fgfr1*. After E9.5 *Fgfr2* is expressed throughout the ventral midline of the MHR and could then take over Fgf signaling in this region. Indeed, no gap in the expression of Fgf signaling targets was seen at E10.5 and E13.5, suggesting a rescue of Fgf signaling by Fgfr2 and/or Fgfr3 in this initial stripe that has been reported to be negative for exression of *Pea3* and *Erm* at earlier stages. It is furthermore conceivable that the loss of a proliferating cell population that is present at the mid-/hindbrain boundary contributes to the defects observed in the $En1^{Cre/+}$ $Fgfr1^{l/l}$ mutant mice. This pool of slowly proliferating cells is also located in the area in which *Fgf8* is expressed at the mid-/hindbrain boundary (Trokovic et al., 2005) and in which neither *Fgfr2* nor *Fgfr3* are expressed at early stages and might therefore exclusively depend on Fgf signaling by Fgfr1. Indeed, loss of *Fgfr1* in the MHR has been reported to lead to a loss of the population of slowly proliferating cells at the mid-/hindbrain boundary (Trokovic et al., 2005). However, although this reduction in slowly proliferating cells might contribute to the loss of 5-HT neurons in anterior r1, it obviously did not affect the mDA neurons, which are present in normal numbers. A compensatory mechanism after loss of functional *Fgfr1* transcript in the $En1^{Cre/+}$ $Fgfr1^{l/l}$ mutants might have been an upregulation of the expression of *Fgfr2* and *Fgfr3* in the MHR. Interestingly, the expression of *Fgfr2* and *Fgfr3* in the MHR was similar in $En1^{Cre/+}$ $Fgfr1^{l/l}$ mutants and wildtype mice. Also in $En1^{Cre/+}$ $Fgfr2^{l/l}$ mutant mice no changes in the expression of the other Fgf receptors were observed (Blak et al., 2007), suggesting that there is no crossregulation between the Fgf receptors in the MHR. Nevertheless, overlapping expression of *Fgfr1* and *Fgfr2* in the ventral MHR of wildtype animals and the sustained activation of targets of Fgf signaling and maintenance of ventral tissue in the $En1^{Cre/+}$ $Fgfr1^{l/l}$ mutants suggested an functional overlap of the Fgf receptors in the developing MHR.

3.3 An allelic series of conditional mutants for *Fgfr1* and *Fgfr2* reveals redundant functions in MHR development

One way to understand the individual contribution of Fgfr1 and Fgfr2 to MHR development was to generate an allelic series of conditional knock outs for *Fgfr1* and/or *Fgfr2*. The resulting single and compound mutants were analyzed for morphological and histological changes in the MHR to find out whether cumulative defects arise with increased loss of Fgf receptor alleles.

The morphological and histological analysis of an allelic series of conditional mutants for *Fgfr1* and *Fgfr2* in the MHR showed that tissue is lost in regions in which no or only low

numbers of Fgf receptor alleles are present. Mutants in which only one allele of *Fgfr1* or both alleles of *Fgfr2* are inactivated in the MHR, i.e. *En1*$^{Cre/+}$ *Fgfr1*$^{l/+}$ or *En1*$^{Cre/+}$ *Fgfr2*$^{l/l}$ respectively, do not show any tissue loss or defects in histology, probably because at least one functional Fgf receptor allele remains expressed throughout the MHR. In *En1*$^{Cre/+}$ *Fgfr1*$^{l/+}$ *Fgfr2*$^{l/l}$ compound mutant mice, which carry only one functional allele of *Fgfr1* in the MHR, defects with variable severity arise in the cerebellum. This might be related to the fact that *Fgfr2* is expressed in the caudal part of the cerebellar anlage from E12.5 on (Blak et al., 2005) and absence of sufficient Fgf receptor expression in the caudal cerebellar anlage could lead to reduced production or differentiation of granule cells which would later migrate along the caudo-rostral axis to build the anterior cerebellar folia (Ryder and Cepko, 1994; Chizhikov and Millen, 2003; Sgaier et al., 2005). Differences in the morphology of cerebellar foliation were reported between several different mouse strains (Inouye and Oda, 1980; Airey et al., 2001). Therefore, genetic modifiers that might be active in some of the mice that were obtained from the mixed breedings used to generate the compound mutant animals might have had an impact on the variable phenotypes observed in the *En1*$^{Cre/+}$ *Fgfr1*$^{l/+}$ *Fgfr2*$^{l/l}$ mutants. Interestingly, the only defect in the *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ *Fgfr2*$^{l/+}$ mutants that was observed in addition to what I have found in the *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ mutants was a precocious downregulation of *Fgf8* and *Wnt1* in the ventral midbrain. No other additional changes were found in patterning, neuronal specification or in activation of Fgf target genes in the MHR of these mutants. However, the analysis of these mutants was not very detailed due to time constraints and further differences might become obvious in future studies on the *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ *Fgfr2*$^{l/+}$ mutants. For example, Fgf signaling could be reduced at E13.5 in these mutants, cell death and proliferation could be changed and further genes involved in Wnt signaling might be regulated differently. *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ *Fgfr2*$^{l/l}$ double knock out mice do not survive after birth and lose all mid-/hindbrain tissue except for the posterior pretectal area which also can be explained by expression of the Fgf receptors in the MHR, as *Fgfr3* is expressed in the most anterior dorsal midbrain from which the pretectum is formed. A summary of the correlation between genotypes and phenotypes in this allelic series of conditional Fgf receptor knock outs in the MHR is given in table 1. In a joint effort with the group of Juha Partanen in Finland, triple knock out mice for the Fgf receptors 1-3 in the MHR were generated and analyzed. In these mice the whole MHR, including the pretectum, is lost (Saarimäki-Vire et al., in press). Altogether, the phenotypes of the above mentioned mutants corroborate the hypothesis that the Fgf receptors are functionally redundant in the generation of tissue in the MHR (compare Fig. 39).

genotype	*En1*$^{Cre/+}$ *Fgfr1*$^{l/+}$	*En1*$^{Cre/+}$ *Fgfr2*$^{l/l}$	*En1*$^{Cre/+}$ *Fgfr1*$^{l/+}$ *Fgfr2*$^{l/l}$	*En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$	*En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ *Fgfr2*$^{l/+}$	*En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ *Fgfr2*$^{l/l}$
defects	none	none	foliation in vermis/loss of vermis, IC misplaced	loss of vermis, loss of IC, changes in VTA and DR	loss of vermis, loss of IC, changes in VTA and DR, ventral Wnt1 expression lost	loss of all MHR structures except pPT
	mild →					**severe**

Table 1. Summary of the defects observed in an allelic series of conditional knock outs for *Fgfr1* and *Fgfr2*. Defects become more severe with increasing loss of Fgf receptor alleles. Loss of *Fgfr1* has an higher impact than loss of *Fgfr2*.

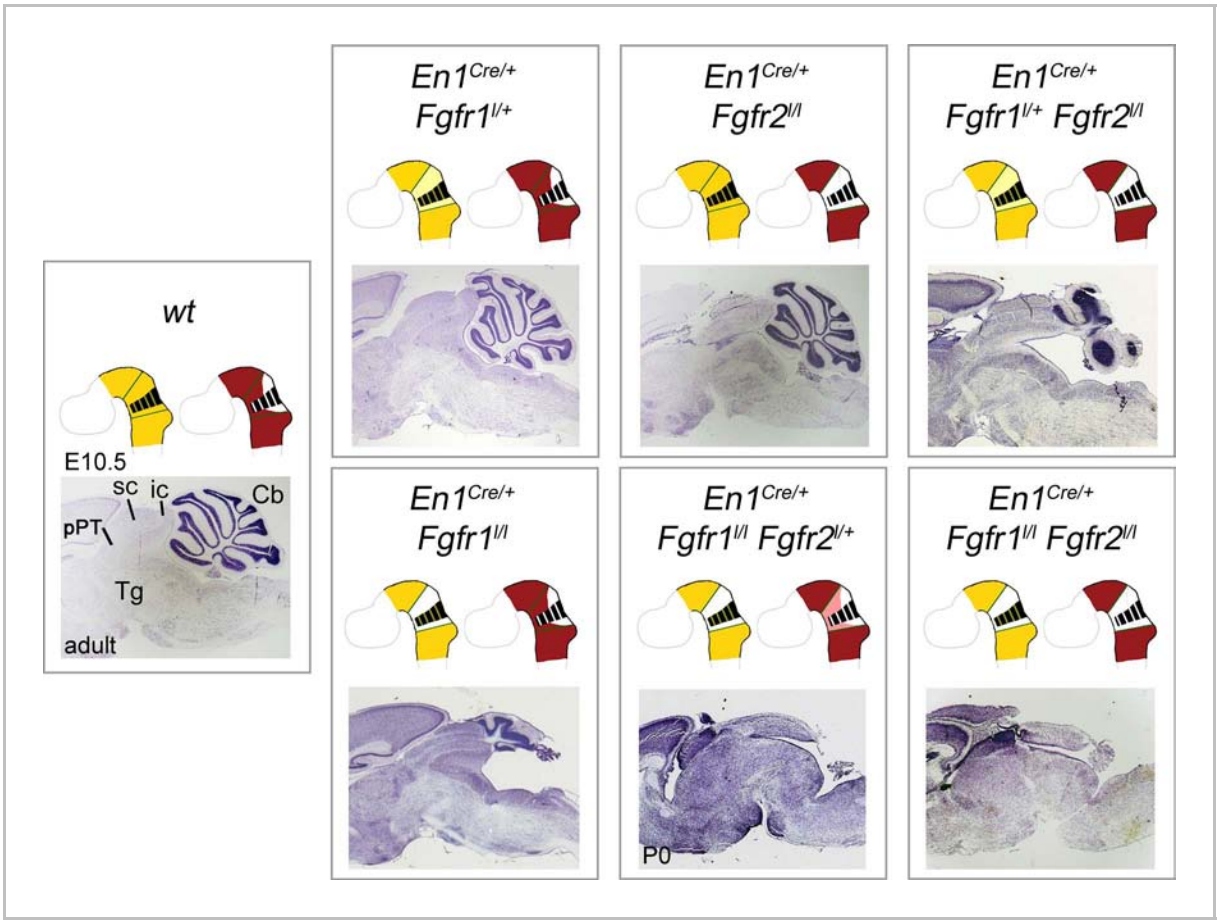

Fig. 39 Compilation showing the relation between the expression and number of alleles for *Fgfr1* and *Fgfr2* in the MHR and the resulting morphology in an allelic series of conditional mutants for *Fgfr1* and *Fgfr2*. Schematic representations of *Fgfr1* and *Fgfr2* expression in the E10.5 MHR are given above pictures of sagittal sections from adult or P0 mutants of the corresponding genotype. *Fgfr1* is painted in orange and *Fgfr2* is given in dark red in the schematic drawings. Dark colors indicate expression of two Fgf receptor alleles, light colors expression of only one allele of the

corresponding Fgf receptor. Deletion of one Fgfr allele does not lead to morphological defects (*En1*$^{Cre/+}$ *Fgfr1*$^{l/+}$). The inactivation of two Fgfr alleles leads to loss of tissue in regions in which a second *Fgfr* is not expressed (dorsal defects in *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$, no defects in *En1*$^{Cre/+}$ *Fgfr2*$^{l/l}$ mutants). Animals which only carry one functional allele of *Fgfr1* in the MHR (*En1*$^{Cre/+}$ *Fgfr1*$^{l/+}$ *Fgfr2*$^{l/l}$) show mild to intermediate defects in dorsal tissues. Correspondingly, animals with only one active allele of *Fgfr2* left (*En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ *Fgfr2*$^{l/+}$) have severe defects in dorsal tissues, where no remaining Fgf receptor is expressed. The most severe deletion of dorsal as well as ventral tissue is found in mutants with complete inactivation of *Fgfr1* and *Fgfr2* in the MHR (*En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ *Fgfr2*$^{l/l}$). pPT: posterior Pretectum; SC: Superior Colliculus; IC: Inferior Colliculus; Cb: Cerebellum; Tg: Tegmentum.

3.4 Conclusion

The absence of the compartment boundary between the midbrain and hindbrain in *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ embryos, as well as the changes observed in ventral patterning and in development of the mDA and 5-HT systems are specific consequences of the loss of *Fgfr1* in the MHR of these mutants. It is interesting that Fgf signaling is rescued in the ventral MHR of the *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ embryos at E10.5 and that other neuronal populations in this region are not affected. The specificity of the observed defects is likely to depend on the exclusive expression of *Fgfr1* within the *Fgf8* positive tissue during early MHR development. In conjunction with the results from the expression study of *Fgfr1-3* in the MHR and with the findings out of the analysis of an allelic series of conditional mutants for *Fgfr1* and *Fgfr2*, this supports the idea of functional redundancy of the Fgf receptors in MHR development. A more detailed analysis and comparison of defects in *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ and *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ *Fgfr2*$^{l/+}$ mutants could further improve the understanding of dosage dependency on Fgf receptors and of Fgf receptor specific functions in the developing MHR of mice.

4 Materials and Methods

4.1 Materials

4.1.1 Suppliers of chemicals and consumables

Standard chemicals were bought from Sigma-Aldrich or Merck. Enzymes and polymerases were obtained from Roche. Components used for synthesis of southern probes were bought from Amersham. Radioactive 35S-rUTP and 32P-dCTP were obtained from Amersham. Plasticware was ordered from Carl Roth GmbH, Eppendorf, StarLab, Polysiences and Greiner. Glassware was from Schott.

4.1.2 Laboratory equipment

Microm HM 355 S rotation microtome
Microm HM 560 cryo microtome
Leica HI 1210 waterbath
Adamas instrument BV SW 85 slide warmer
Leica EG 1160 paraffin embedding station
Heidolph instruments Polymax 1040 wave shaker
Eppendorf thermomixer comfort
Eppendorf Mastercycler gradient
Eppendorf BioPhotometer
NeoLab Thermo EC 250-90 power supply
Sharp R-937 IN microwave oven
PeqLab Gelsystem Mini L
Sartorius LC 6201S scale
WTW inolab 720 pH meter
New Brunswick scientific innova 4230 refigerated incubation shaker
Haereus Biofuge pico
Memmert UM 400 oven
AGFA Curix 60 developing machine
Berthold LB122 radiation monitor

Hidex Triathler liquid scintillation counter
Herolab gel documentation system
IKA Labortechnik RCT basic heater
Thermo Hybaid Shake'n'stack hybridization oven
Leica KL 1500 light source
Zeiss Axiocam MRC digital camera
Zeiss Axioplan 2 imaging microscope
Zeiss Stemi SV6 stereomicroscope
Stratagene UV Stratalinker 1800 crosslinker
BENDA Laborgeräte N36 UV table

4.1.3 Buffers and solutions

Standard buffers used were prepared according to Sambrook and Fritsch, "Molecular Cloning", third edition, 2001, Cold Spring Harbor, New York.

4.1.3.1 Special buffers and solutions used for in-situ:

Hyb-Mix
- 50% formamide
- 20mM Tris-HCl, pH 8.0
- 300mM NaCl
- 5mM EDTA, pH 8.0
- 10% dextrane sulphate
- 0.02% Ficoll 400
- 0.02% PVP40
- 0.02% BSA
- 0.5mg/ml tRNA
- 0.2mg/ml carrier DNA, acid cleaved
- 20 mM DTT

Proteinase K buffer
- 0.5M Tris-HCl, pH 7.6
- 50mM EDTA, pH 8.0

NTE

2.5M NaCl

50mM Tris-HCl, pH 8.0

25mM EDTA, pH 8.0

Hybridization chamber fluid

50% formamide

2x SSC

4.1.3.2 Special buffers and solutions used for immunohistochemistry:

Blocking solution

5% FCS

0.1% PBT

ABC working solution

1:300 Reagent A

1:300 Reagent B

in PBS, prepare at least 30 min before use

DAB working solution

0.05% DAB

0.1M Tris-HCl

add 15µl H_2O_2 immediately before use

4.1.3.3 Special buffers and solutions used for Nissl staining:

Cresyl violet solution

0.5% Cresyl violet

0.6mM sodium acetate

50mM acetic acid

adjust to pH 3.5

4.1.4 Kits

4.1.4.1 Kits used for purification of nucleic acids

RNeasy Mini Kit (Qiagen)
Wizard Genomic DNA Purification Kit (Promega)
Pure Yield Plasmid Midiprep System (Promega)
QIAquick PCR Purification Kit (Qiagen)
QIAquick Gel Extraction Kit (Qiagen)

4.1.4.2 Kits used for immunohistochemistry

Vectastain Elite ABC Kit (Vector Labs)

4.1.5 Antibodies

4.1.5.1 Primary antibodies used

Antibody	Supplier	Dilution	2nd AB dilution	Embedding
Rabbit Anti TH	Pel-Freez	1:10000	1:300	Cryo
Rat Anti Dat	Chemicon	1:1000	1:300	Cryo
Rabbit Anti 5-HT	Cappel	1:1000	1:300	Cryo
Rabbit Anti Parvalbumin	Swant	1:2000	1:300	Cryo
Rabbit Anti Calbindin	Swant	1:5000	1:300	Cryo

Table 2. Primary antibodies used for immunohistochemistry.

4.1.5.2 Secondary antibodies used

Antibody	Supplier
Biotin-SP-conjugated goat anti rabbit IgG (H+L)	Jackson Immuno research
Biotin-SP-conjugated goat anti rat IgG (H+L)	Jackson Immuno research

Table 3. Secondary antibodies used for immunohistochemistry.

4.1.6 Enzymes and RNA polymerases used for synthesis of in-situ probes

probe template	enzyme for linearization	RNA polymerase	provided by	Accession number
Dat	Hind III	T7	C. Brodski	BC054119
DBH	Xho I	Sp6	S. Laaß	NM_138942
Emx2	Nco I	Sp6	A. Simeone	NM_010132
En1	Hind III	T7	A. Joyner	NM_010133
Fgf8	Pst I	T7	S. Martinez	NM_010205
Fgfr1	Nde I	T7	R.Lauster	BC010200
Fgfr2	BamH I	T7	J.M. Revest	BC110661
Fgfr3	Hind III	T7	D. Ornitz	BC053056
Fzd3	Sac I	T7	T. Fischer	NM_021458
Fzd6	EcoR V	Sp6	T. Fischer	NM_008056
Gbx2	Hind III	T7	G.R. Martin	L39770
Lmx1b	Xho I	Sp6	C. Brodski	AF078166
Mash1	Xba I	Sp6	F. Guillemot	NM_008553
MKP3	Sal I	T7	J. A. Belo	BC003869
Nkx2.2	Not I	T7	D. Hartigan	NM_010919
Nkx2.9	Hind III	T7	E. Puelles	NM_008701
Nurr1	Hind III	T7	C. Brodski	NM_013613
Otx2	EcoR I	Sp6	A. Simeone	NM_144841
Pet1	BamH I	T7	H. Pfaar	NM_153111
Pitx3	Not I	Sp6	J. Guimera	NM_008852
Pou4f1	Not I	Sp6	A. Simeone	S69350
Sef1	Nco I	Sp6	R. Friesel	AF459444
Sert	Hind III	T7	C. Brodski	AF013604
Shh	Hind III	T3	A. McMahon	BC063087
Spr1	EcoR I	T7	G.R. Martin	NM_011896
TH	SpH I	Sp6	J. Guimera	NM_009377
VAChT	Not I	Sp6	C. Brodski	NM_021712
Wnt1	Hind III	T7	C. Brodski	NM_021279
Wnt5a	EcoR I	Sp6	A. McMahon	NM_009524

Table 4. Listing of templates used for the generation of in-situ probes. Restriction enzymes and RNA polymerases used for the synthesis of the probes are given.

4.1.7 Primers used for PCR

PCR	Oligo name	Sequence	expected product length (wt / mutant)
Cre	Cre 5'	AATCTCCCACCGTCAGTACG	
	Cre 3'	CGTTTTCTGAGCATACCTGGA	- / 500
Fgfr1	7-8del5	CCCCATCCCATTTCCTTACCT	
	7-8del3	TTCTGGTGTGTCTGAAAACAGCT	150 / 200
Fgfr2	Fgfr2fwd	CTAGGCCAGCTGGACCAGAC	
	Fgfr2rev	CGTTCTCTGATGGGCCATTG	750 / 1000

Table 5. Oligonucleotides used as primers for PCR.

4.1.8 Bacteria used for Plasmid amplification

DH5α

4.1.9 Mouse lines

CD1
C57BL6/J
Fgfr1$^{lox/lox}$ (Partanen J.)
Fgfr2$^{lox/lox}$ (Sendtner M.)
En1$^{Cre/+}$ (Wurst W.)

En1$^{Cre/+}$ knock in mice (Kimmel et al., 2000) were crossed to *Fgfr1*$^{l/l}$ homozygous females (Trokovic et al., 2003) and male offspring carrying the *Cre* allele was bred to female offspring that did not carry the *Cre* allele to obtain the *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ mutants. *En1*$^{Cre/+}$ *Fgfr2*$^{l/l}$ mice were obtained by analogous breedings. A line of *Fgfr1*$^{l/l}$ *Fgfr2*$^{l/l}$ mice was established by intercrossing the single mutant *Fgfr1*$^{l/l}$ and *Fgfr2*$^{l/l}$ lines and inbreeding of double heterozygous offspring from the F1 generation. *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ males were crossed with *Fgfr1*$^{l/l}$ *Fgfr2*$^{l/l}$ females (Blak et al., 2007) to obtain the *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ *Fgfr2*$^{l/+}$ compound mutants. Crossing of *En1*$^{Cre/+}$ *Fgfr2*$^{l/l}$ males with females from the *Fgfr1*$^{l/l}$ *Fgfr2*$^{l/l}$ line provided *En1*$^{Cre/+}$ *Fgfr1*$^{l/+}$ *Fgfr2*$^{l/l}$ animals. Breeding of males with this genotype with female *Fgfr1*$^{l/l}$ *Fgfr2*$^{l/l}$ mice provided *En1*$^{Cre/+}$ *Fgfr1*$^{l/l}$ *Fgfr2*$^{l/l}$ double homozygous embryos.

4.2 Methods

4.2.1 Bacterial cultures

The medium used for bacterial culture was Luria Bertani (LB) with 50mg/ml of Ampicillin. Clones were picked directly from a plate and inoculated in 250ml of medium. Overnight cultures (16h) of bacteria were kept in a shaker at 37°C.

4.2.2 DNA extractions

4.2.2.1 Plasmid DNA extraction

Plasmid DNA was extracted from bacterial cultures according to the Promega Pure Yield Plasmid Midiprep System kit protocol. DNA was resuspended in 300µl preheated water.

4.2.2.2 Genomic DNA extraction with kit

Isolation of genomic DNA from mouse tails and embryonic tissue was done according to the Promega wizard genomic DNA purification kit protocol. Pellets were resuspended for 1h in 300µl 10 mM Tris-HCl (for tail DNA) or 100 µl Tris-HCl (for embryonic DNA) at 65°C.

4.2.3 DNA digest

DNA was digested with a ten fold excess of enzyme, i.e. 10U / 1µg DNA. The total volume of the reaction was adjusted to ten times the volume of the enzyme solution added.

4.2.4 Agarose gel electrophoresis

Agarose gels ranged in concentration from 0.8% to 2%, depending on the size of the nucleic acids. Gels for visualization of PCR products were prepared using TAE buffer, gels for southern blot were 0.8% Agarose in TBE. Photographs of the separated nucleic acids were taken using a Herolab gel documentation system with a UV light table.

4.2.5 PCR

PCR reactions were set up according to table 6.

Fgfr1/Cre		Fgfr2	
compound	amount	compound	amount
water	14,5	water	14,5
10x buffer	2	10x buffer	2
dNTPs	0,5	dNTPs	0,5
primer	0,5	primer	0,8
Taq-Pol.	0,5	Taq-Pol.	0,5

Table 6. Reaction mixtures used for PCR of genomic fragments of Cre recombinase, Fgfr1 or Fgfr2.

Conditions for the various PCR reactions are given in table 7.

Protocol	Fgfr1 / Cre	Fgfr2
annealing	54°C, 45''	58°C, 45''
extension	72°C, 30''	72°C, 1'30''
# cycles	35	35

Table 7. Conditions for PCR reactions to amplify genomic fragments of Cre recombinase, Fgfr1 or Fgfr2.

4.2.6 Southern blot

4.2.6.1 Extraction and Preparation of DNA

Approximately 10µg of DNA was digested o.n. with a 10 fold excess of Hind III (i.e. 10U / µg of DNA). Loading buffer was added and the digest was heated to 65°C for 15' before snap cooling on ice.

4.2.6.2 Gel electrophoresis and blotting

35µl of the DNA solution were loaded onto an agarose gel and run o.n. at low voltage. A picture of each gel was taken, to judge the quality of the digested DNA. The following day the gel was put into denaturation solution for 30' and washed twice in neutralization solution for 15'. Then a horizontal transfer stack for upward capillary transfer was built and left o.n. for transfer of the DNA onto a nylon membrane.

4.2.6.3 Treatment of membranes

Transferred DNA was immobilized on the membrane by UV-Light irradiation (2x 1200J) and the blot was prehybridized in Rapid-Hyb mix (Amersham) for 1h at 65°C. Radioactive probe was added to an activity of 1 Mcpm / ml hybridization solution and incubated o.n. at 65°C. The membrane was washed two times in 2x SSC / 0.1% SDS at room temperature for 5' and 2x in 0.5x SSC / 0.1% SDS at 37°C for 15'. After washing the remaining activity of the blot was checked with a beta-counter. Blots with an activity of more than 300 cpm were washed again in 0.5x SSC / 0.1% SDS at 37°C until signal intensity was below 300 cpm. Membranes were then exposed to film for one to three days and developed.

4.2.6.4 Probe synthesis

The *Fgfr1* probe was cut out from plasmid 6.12R with the enzymes Pst I and EcoR I. The probe sequence is located in the 3' of the coding region in the genomic locus of *Fgfr1*. Hybridization to the wt allele of *Fgfr1* recognizes a 9 kb fragment, while the fragment of the mutant allele has a size of 5 kb.
After cutting the digest was run on an 0.8% agarose gel and the excised 800bp fragment was purified using the Qiagen gel extraction kit. Concentration of the purified DNA fragment was determined with a photometer.
10 ng of the probe template were used in the labelling reaction, which was carried out according to the Amersham Rediprime labelling kit protocol. Labelled probes were applied to a Amersham microspin S300 HR column and recovered by centrifugation. The activity of the probe was determined in a liquid scintillation counter.

4.2.7 Dissections

4.2.7.1 Dissection of embryos

Female mice were checked for vaginal plug when breeding. Noon of the day of vaginal plug was considered as embryonic day 0.5 (E0.5). Additional staging of the dissected embryos was done according to "The Atlas of Mouse Development, Kaufman MH, 1992, Academic Press Limited, London. Embryos were isolated from the placenta and put into 4% PFA. Tissue for genotyping was taken from the Amnion at stages before E11.5, or from the trunk of the embryo from E11.5 on.

4.2.7.2 Dissection of brains from postnatal mice

Mice were killed in CO_2 and intracardially perfused with 4%PFA immediately after death. The skull was opened along its sides, bones and meninges were removed and the brain was put into 4% PFA overnight. For use in cryosectioning, brains were put into 20% sucrose the next day and left o.n. before cutting. For use in paraffin sectioning, brains were dehydrated as given below (paraffin embedding).

4.2.8 Histology

4.2.8.1 Fixation and embedding of tissue for paraffin sectioning

Tissue was fixed in 4% PFA at 4°C overnight. Times for dehydration of tissue in an ascending ethanol series with 70%, 96% and 100% ethanol steps depended on the size of the tissue/ stage of the embryo. Incubation in Xylol was controlled by translucence of the tissue, recommended times of incubation are given in table 8. After dehydration, tissues were transferred into Paraffin at 65°C, overnight. On the following day, tissues were embedded in paraffin in peel-a-way embedding molds.
Paraffin embedded tissue was cut at 8µm thickness on a rotation microtome and consecutive sections mounted on series of Superfrost slides.

Stage	4% PFA	Ethanol 70%	Ethanol 96%	Ethanol 100%	Rotihistol or Xylol	Paraffin
E8.5	overnight	5 min	5 min	5 min	3-5 min	overnight
E9.5	overnight	10 min	10 min	10 min	5-7 min	Overnight
E10.5	overnight	10 min	10 min	10 min	10 min	overnight
E11.5	overnight	15 min	15 min	15 min	13 min	overnight
E12.5	overnight	15 min	15 min	15 min	15 min	Overnight
E13.5	overnight	20 min	20 min	20 min	20 min	Overnight
E14-P0	overnight	30 min	30 min	30 min	30 min	overnight

Table 8. Sequence of washing steps for the dehydration of embryonic tissues.

4.2.8.2 Fixation and cutting of tissue for cryosections

Adult animals were perfused and the brain was dissected and put into 4% PFA o.n. at 4°C. Tissues were transferred into 20% Sucrose solution the next day and incubated o.n. at 4°C.

Free floating cryosections were made on a cryo microtome in horizontal or sagittal orientation at a thickness of 40µm and transferred into 6-well plates with PBS (for immediate use) or cryoprotection solution (for temporary storage at 4°C).

4.2.8.3 Cresyl-violet (Nissl) staining

Nissl staining of dried sections was performed according to the protocol in table 9. The times given can vary, depending on the type of tissue and sections used. Careful monitoring of the staining is necessary to obtain optimal results.

step	duration
cresyl violet	2'
water	5'
2x 70% EtOH	2'
2x 96% EtOH	2'
2x 100% EtOH	2'
2x Xylol or Rotihistol	5'

Table 9. Protocol for cresyl violet staining of sections on slides.

After Nissl staining, slides were air dried and covered using the reagents given below.

4.2.8.4 Mounting of slides

Sections on slides were dehydrated in an ascending EtOH series, as described elsewhere. Slides transferred into Xylol were covered with DPX, slides transferred into Rotihistol were covered with Roti-Histokitt.

4.2.9 Immunohistochemistry

4.2.9.1 Immunohistochemistry on paraffin sections

Paraffin sections were treated for immunohistochemistry according to the protocol given below.

1st day:

1. dewax	1 hr	Rotihistol	
2. rehydration	2 x 5 min	Ethanol 100%	
3.	2 x 5 min	Ethanol 96%	
4.	2 x 5 min	Ethanol 70%	
5.	10 min	Aqua dest.	
6. Antigen retrieval	3 min	0,01M NaCitrate	RT
7.	5 min	0,01M NaCitrate	Microwave max.
8.	10 min	0,01M NaCitrate	Cooling at RT
9.	3 min	0,01M NaCitrate	Microwave 360W
10.	20 min	0,01M NaCitrate	Cooling to RT
11. wash	2 x 5 min	PBS	
12. bleach	5 min	0,01% H_2O_2 / PBS	
13. wash	2 x 5 min	PBS	
14. blocking	1 hr	Blocking solution	
15. 1st Antibody	Overnight	1st Antibody solution	4°C

2nd day

1. wash	3 x 5 min	PBS	
2. 2nd antibody	1 hr	2nd antibody solution	
3. wash	3 x 5 min	PBS	
4. ABC complex	30 min	ABC reagent	
5. wash	2 x 5 min	PBS	
6. wash	1 x 5 min	0,1M Tris-HCl	
7. DAB reaction	3 – 30 min	DAB working solution	Check staining to determine duration!
8. wash	2 x 5 min	PBS	
9. dehydration	2 x 5 min	Ethanol 70%	

10.	2 x 5 min	Ethanol 96%	
11.	2 x 5 min	Ethanol 100%	
12.	2 x 5 min	Rotihistol	

4.2.9.2 Immunohistochemistry on cryo sections

Cryo sections were treated for immunohistochemistry according to the protocol given below.

1st day

1. wash	Over night or 2 x 5 min	PBS	Over night if slides were stored in cryoprotection solution
2. destruction of endogenous peroxidases	10 min	0.1% H_2O_2 / PBS	Add H_2O_2 directly before use
3.wash	2 x 10 min	PBS-Triton	
4. blocking	2 hr	Blocking solution	RT
5.1st antibody	Over night	1st antibody solution	4°C

2nd day

1. wash	4 x 15 min	PBS-Triton	
2. 2nd antibody	45 min	2nd antibody-solution	RT
3. wash	4 x 15 min	PBS-Triton	
4. intensifying	45 min	ABC-solution	
5. wash	2 x 15 min	PBS-Triton	
6. wash	2 x 15 min	0.1M Tris-HCl	
6. DAB-staining	2 – 20 min	DAB-working solution	Check intensity under microscope
7. stop staining	3 x 10 min	PBS	
8. mounting			Mount slices on slides

4.2.10 In-situ hybridization on paraffin sections

4.2.10.1 Preparation of template DNA

Plasmid DNA containing the probe sequence was linearized by digestion with the appropriate enzyme. The linearized DNA was then purified according to the Qiagen PCR purification Kit protocol and the concentration was determined by photometry. The concentration was adjusted to 250 ng/µl and aliquots of 8µl were stored at -20°C.

4.2.10.2 Probe synthesis

Probes for in-situ hybridization on paraffin sections were radioactively labelled RNA probes, synthesized according to the setup in table 10.

compound	amount
DEPC-water	6.5µl
10x transcription buffer	3µl
NTP Mix (10mM)	3µl
0,5M DTT	1µl
RNasin	1µl
DNA template (2µg)	8µl
35-S UTP	3µl
RNA Pol.	1µl

Table 10. Reaction mix for the synthesis of RNA probes.

Labelling reactions were carried out at 37°C for 3h. After 1h of incubation another 0.5µl of the appropriate RNA polymerase were added. Subsequently, a 15' DNAse I digest of the DNA template was performed. The labelled probes were then purified according to the Qiagen RNeasy protocol and stored at -20°C for up to two weeks.

4.2.10.3 Pretreatment of Paraffin sections

Before hybridization, Paraffin sections were dewaxed and treated according to the protocol given below.

1. dewax	2 x 15 min	Rotihistol	check dewaxing, time can be elongated
2. rinse	2 x 5 min	100 % Ethanol	
3.	5 min	70 % Ethanol	
4.	3 min	DEPC-H_20	

5.	3 min	PBS/DEPC	
6.	20 min	4 % PFA/PBS	on ice
7.	2 x 5 min	PBS/DEPC	
8.	7 min	20 µg/ml Proteinase K in Proteinase-K-buffer	add 200 µl of Proteinase K (20 mg/ml)
9.	5 min	PBS/DEPC	
10.	20 min	4 % PFA/PBS	same solution from NO 6
11.	5 min	PBS/DEPC	
12.	10 min	200 ml of rapidly stirring 0,1 M triethanolamine-HCl (pH8) (TEA)	add 600 µl acetic anhydride; (for the second add anothertime 600 µl acetic anhydrid)
13.	2 x 5 min	2xSSC	
14.	1 min	60 % Ethanol/DEPC	
15.	1 min	70 % Ethanol/DEPC	
16.	1 min	95 % Ethanol/DEPC	
17.	1 min	100 % Ethanol	
18. Air dry (dust free !)			

4.2.10.4 Hybridization

Slides were then left to dry and subsequently prehybridized with Hyb-mix for one hour at 57°C. Probes were diluted to an activity of 7Mcpm/100µl in Hyb-Mix and denatured at 90°C. After snap cooling on ice, 100µl of the Hyb-mix containing the probe were added to each slide. Slides were covered and left o.n. in a humid chamber (containing chamber fluid) at 57°C.

4.2.10.5 High stringency washes

The next day, the slides were uncovered and washed according to the following protocol:

1.	4 x 5 min	Rt	4xSSC	
2.	20 min	37 °C	NTE (20µg/ml RNaseA)	add 500 µl RNaseA(10mg/ml) to 250 ml of NTE
3.	2 x 5 min	Rt	2xSSC/1 mM DTT	50 µl of 5 M DTT/250 ml
4.	10 min	Rt	1xSSC/1 mM DTT	50 µl of 5 M DTT/250 ml

5.	10 min	Rt	0,5xSSC/1mM DTT	50 µl of 5 M DTT/250 ml
6.	2 x 30 min	64 °C	0,1xSSC/1 mM DTT	50 µl of 5 M DTT/250 ml
7.	2 x 10 min	Rt	0,1xSSC	
8.	1 min	Rt	30 % Ethanol in 300 mM NH_4OAc	
9.	1 min	Rt	50 % Ethanol in 300 mM NH_4OAc	
10.	1 min	Rt	70 % Ethanol in 300 mM NH_4OAc	
11.	1 min	Rt	95 % Ethanol	
12.	2 x 1 min	Rt	100 % Ethanol	

4.2.10.6 Exposure and developing of the slides

After drying, labelled slides were exposed to x-ray film for three days and the quality of the hybridization was judged. Subsequently, the slides were dipped in Kodak NTB photoemulsion and exposed in dark boxes for up to 6 weeks at 4°C. Subsequent to the exposure, slides were developed for 3' in developer solution (Kodak D19 developer), washed 30'' in tap water and fixed for 6' in Kodak fixer. Slides were then rinsed with tap water for 20' and subsequently dried. Before counterstaining with Cresyl violet, photoemulsion was scratched off from the backside of the slides.

4.2.11 Microscopy

Optical evaluation of tissues from ISH or immunohistochemistry was done on a Zeiss Axioplan 2 imaging microscope or Zeiss Stemi SV6 binocular. Pictures were taken at a resolution of 1300 x 1030 pixel with a Zeiss AxioCam MRC camera using the Axiovision 4.6 software. Counting of cells and area determination were done on using the Stereoinvestigator software in optical fractionator or Cavalieri mode, respectively.

4.2.12 Statistics

Statistical analysis of results from the stereological experiments was performed using ANOVA and students t-test.

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6 Appendix

6.1 List of abbreviations

'	minutes
"	seconds
%	percent (weight per volume or volume per volume)
(m)DA	(midbrain) dopaminergic neurons
°C	degree Celius
µ	micro (10^{-6})
µl	microliter
µm	micrometer
5-HT	serotonin or serotonergic
a/p	antero-posterior axis
bp	base pair
BSA	bovine serum albumin
Cb	Cerebellum
CNS	central nervous system
cpm	counts per minute
d/v	dorso-ventral axis
DAB	diaminobenzidine
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DR	dorsal Raphe nucleus
DTT	dithiothreitol
E	embryonic day
EDTA	ethylenediamine tetraacetate
EtOH	ethanol
FB	forebrain
FCS	fetal calf serum
Fig.	figure
GABAergic	neurons producing the neuro-transmitter gamma-aminobutyric acid
h or hr	hour
H_2O_2	hydrogen peroxide
HB	hindbrain
HCl	hydrogen chloride
IC	inferior colliculi
III	nucleus of 3rd cranial nerve
IV	nucleus of 4th cranial nerve
J	Joule
LB	Luria Bertani medium
LC	locus coeruleus
m	milli (10^{-3})
m/l	medio-lateral axis
MB	midbrain
Mcpm	million counts per minute
Mes	mesencephalon
Met	metencephalon
mg	milligram
MHO	mid-/hindbrain organizer
MHR	mid-/hindbrain region
min	minutes
ml	milliliter
mM	millimolar
MR	medial Raphe nucleus
Myel	myelencephalon
n	nano (10^{-9})
Na	sodium
NaCl	sodium chloride
ng	nanogram
NTE	sodium-Tris-EDTA buffer
o.n.	over night
P	postnatal day (P0 is the day of birth)
PBS	phosphate buffered saline
PBT	phosphate buffer containing Tris
PCR	polymerase chain reaction
PFA	para-formaldehyde
pH	$-\log_{10}$ of the concentration of hydrogen ions (pondus hydrogenii)
Pol.	polymerase
pPT	posterior pretectum
PVP	polyvinylpyrrolidone
r	rhombomere
RT	room temperature
SC	superior colliculi
SDS	sodium dodecyl sulfate
SN(pc)	substantia nigra (pars compacta)
SSC	saline-sodiumcitrate buffer
TAE	Tris-acetate-EDTA buffer
TBE	Tris-borate-EDTA buffer
Tel	telencephalon
Tris	tris(hydroxymethyl)aminomehtane
tRNA	transfer ribonucleic acid
U	unit (of enzyme activity)
UTR	untranslated region
UV	ultraviolet
VTA	ventral tegmental area
wt	wildtype

6.2 Gene symbols

Dat	dopamine reuptake transporter (Slc6a3)
DBH	dopamine beta hydroxylase
Emx2	empty spiracles homolog homebox 2
En	engrailed homeobox transcription factor
Erm	Ets related molecule PEA3-like (Etv5)
Fgf	fibroblast growth factor
Fgfr	Fgf receptor
Fzd	frizzled homolog
Gata	GATA-binding protein
Gbx2	gastrulation brain homeobox transcription factor 2
Girk2	G-protein coupled inward rectifying potassium channel 2 (Kcnj6)
Lmx1a/b	LIM domain homeobox transcription factor 1alpha and 1beta
Mash1	mouse achaete scute homolog 1
MKP3	MAP kinase phosphatase 3 (Dusp6)
Nkx2.2/2.9	NK2 transcription factor related, locus 2 and 9
Nurr1	Nuclear receptor related protein 1 (Nr4a2)
Otx2	orthodenticle like homeobox transcritpion factor 2
Pax	Paired like homeobox transcription factor
Pea3	polyomavirus enhancer activator 3 (Etv4)
Pet1	PC12 ETS factor 1 (FEV)
Pitx3	paired like homeodomain transcription factor 3
Pou4f1	Pou domain family 4 factor 1 (Brn3a)
Raldh1	retinaldehyde dehydrogenase 1 (Aldh1a1)
Sef1	Similar expression to Fgf 1 (Il17rd)
Sert	serotonin reuptake transporter (HTT/Slc6a4)
Shh	sonic hedgehog
Spr1	Sprouty homolog 1
TH	tyrosine hydroxylase
VAChT	vesicular acetylcholine transporter (Slc18a3)
Wnt	wingless-type MMTV integration site

6.3 Curriculum vitae

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6.4 List of publications

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* equal contribution

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