Fgf3 is crucial for the generation of monoaminergic cerebrospinal fluid contacting cells in zebrafish

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ABSTRACT
In most vertebrates, including zebrafish, the hypothalamic serotonergic cerebrospinal fluid-contacting (CSF-c) cells constitute a prominent population. In contrast to the hindbrain serotonergic neurons, little is known about the development and function of these cells. Here, we identify fibroblast growth factor (Fgf3) as the main Fgf ligand controlling the ontogeny of serotonergic CSF-c cells. We show that fgf3 positively regulates the number of serotonergic CSF-c cells, as well as a subset of dopaminergic and neuroendocrine cells in the posterior hypothalamus via control of proliferation and cell survival. Further, expression of the ETS-domain transcription factor etv5b is downregulated after fgf3 impairment. Previous findings identified etv5b as critical for the proliferation of serotonergic progenitors in the hypothalamus, and therefore we now suggest that Fgf3 acts via etv5b during early development to ultimately control the number of mature serotonergic CSF-c cells. Moreover, our analysis of the developing hypothalamic transcriptome shows that the expression of fgf3 is upregulated upon fgf3 loss-of-function, suggesting activation of a self-compensatory mechanism. Together, these results highlight Fgf3 in a novel context as part of a signalling pathway of critical importance for hypothalamic development.

KEY WORDS: Fgf-signalling, Serotonin, Dopamine, Hypothalamus, Central nervous system

INTRODUCTION
Serotonin (5-hydroxytryptamine, 5-HT) is an ancient signalling molecule present in the nervous system of animals from cnidian and bilaterian lineages (Hay-Schmidt, 2000; Kass-Simon and Pierbon, 2007; Moroz and Kohn, 2016; Stach, 2005). Accordingly, 5-HT modulates a variety of physiological processes and behaviours in most animals (e.g. Curran and Chalasani, 2012; Fossat et al., 2014; Gillette, 2006; Lucki, 1998; Trowbridge et al., 2011). In placental mammals, serotonergic neurons are, with a few potential exceptions (Ballion et al., 2002; Ugrumov et al., 1989), uniquely located in the raphe nuclei of the hindbrain (Deneris and Gaspar, 2018). In contrast, additional serotonergic cell populations are found in the forebrain and spinal cord in cartilaginous and bony fish, amphibians, reptiles and monotremes (Lillesaar, 2011; Montgomery et al., 2016). Most of these non-raphe cell populations are truly serotonergic as they contain not only 5-HT, but also proteins required for 5-HT metabolism, packaging and transport. To what extent the development of different populations of serotonergic cells is controlled by the same paracrine signals and gene regulatory networks to ultimately express the mature serotonergic phenotype (i.e. capacity to synthesise 5-HT) is still unclear.

The ontogeny as well as the regulatory transcriptional networks of raphe serotonergic neurons are well described (Deneris and Gaspar, 2018; Deneris and Wyler, 2012; Flames and Hobert, 2011; Kiyasova and Gaspar, 2011; Lillesaar et al., 2007, 2009; McLean and Fitch, 2004; Horton et al., 2005; Teraoka et al., 2004). In contrast, little is known about the development of the remaining populations in the central nervous system. In zebrafish, the hypothalamus contains by far the highest number of serotonergic cells (Lillesaar, 2011). These cells are small, bipolar cells with one thick process contacting the ventricle suggesting that they can communicate over longer distances via the cerebrospinal fluid (CSF), and the other process projecting locally in the brain (Lillesaar, 2011; Sano et al., 1983; Vigil et al., 2004). Anatomically, these cells are further separated into three clusters in teleost fish; the anterior, intermediate (i.) and posterior (p.) paraventricular organ clusters (Ekström et al., 1985; Kaslin and Panula, 2001), of which the latter is located around the posterior recess, a ventricular structure found in the hypothalamus of teleosts (Xavier et al., 2017). Overlapping expression of tph1a, slc6a4b, ddc, mao and vmat2 (Anichtchik et al., 2006; Lillesaar et al., 2007; Norton et al., 2008; Sallinen et al., 2009; Yamamoto et al., 2011) shows an active 5-HT metabolism in the region. Notably, serotonergic and dopaminergic cells are largely intermingled populations in the hypothalamus (Filippi et al., 2009; Kaslin and Panula, 2001; McLean and Fitch, 2004; Xavier et al., 2017; Yamamoto et al., 2010, 2011).

Progenitors giving rise to neuronal and glial precursors of the developing hypothalamus are located at the ventricular zone (Duncan et al., 2016; Xie and Dorsky, 2017). Eventually the progenitors exit the cell cycle, migrate laterally and continue differentiation. Lineage specific combinations of transcription factors control the fate of the precursors (Biran et al., 2015; Burbridge et al., 2016; Muthu et al., 2016; Ware et al., 2014; Xie and Dorsky, 2017). The precise set of paracrine molecules and transcription factors required in time and space for each cell type is still a matter of investigation, as is the extent of evolutionary conservation. In zebrafish, the location of the serotonergic cells in...
the posterior hypothalamus suggests that their fate is favoured by posteriorising signals such as Wnt and Fgf (Kapsimali et al., 2004; Xie and Dorsky, 2017), and inhibited by anteriorising signals such as late Shh expression (Mathieu et al., 2002; Muthu et al., 2016). Indeed, Fgf ligands, receptors and downstream targets are expressed in the posterior zebrafish hypothalamus (Bosco et al., 2013; Herzog et al., 2004; Jackman et al., 2004; Liu et al., 2013; Reifers et al., 1998; Topp et al., 2008). Similarly to raphe serotonergic neurons, hypothalamic serotonergic CSF-c cells along with dopaminergic cells depend on Fgf-signalling during development (Bosco et al., 2013; Koch et al., 2014; Teraoka et al., 2004). Furthermore, Etv5b, a member of the ETS-domain transcription factor family that is a direct downstream target of Fgf-signalling (Ornitz and Itoh, 2015; Raible and Brand, 2001; Roussigné and Blader, 2006), regulates the proliferation of serotonergic progenitors (Bosco et al., 2013).

Here, we are focusing on the hypothalamic serotonergic cells of zebrafish, a frequently used model organism in biomedical research. Using three different approaches for genetic manipulation, we identify Fgf3 as the main Fgf ligand critical for development of serotonergic as well as dopaminergic CSF-c cells and arginine vasopressin (avp)-expressing cells located in the posterior hypothalamus. Further, based on sequencing of the transcriptome of microdissected hypothalami we identify genes belonging to the Fgf-signalling pathway that are expressed in the developing hypothalamus, and demonstrate mild alterations of Fgf-signalling after impairment of fgf3. With this information we acquire a better knowledge about the signalling networks promoting the ontogeny of central serotonergic cells.

**RESULTS**

fgf3 is expressed in the developing hypothalamus and spatially correlates with the location of putative serotonergic CSF-c cells

Among the described Fgf ligands, fgf3 exhibits the most prominent distribution in the posterior hypothalamus (Herzog et al., 2004; Jackman et al., 2004; Liu et al., 2013; Reifers et al., 1998; Topp et al., 2008) presumably where serotonergic precursor cells are located, therefore rendering Fgf3 a likely candidate regulating the development of hypothalamic serotonergic CSF-c cells. To explore the dynamics of hypothalamic fgf3 expression we performed a spatio-temporal expression analysis. We could confirm presence of fgf3 transcripts in the developing hypothalamus (Fig. 1). More specifically, transcripts were first detectable at 20 somites in the hypothalamic primordium (Fig. 1A–D). This expression was maintained until 30 h post fertilisation (hpf) (Fig. 1E,F). From 36 hpf and onwards the signal was restricted to the posterior hypothalamus (Fig. 1G–J). Further, expression of fgf3 is restricted to cells located medially at the ventricle (arrowheads), while dusp6 and etv5b are present laterally, in cells around the posterior recess.

![Fig. 1. RNA in situ hybridisation reveals fgf3 expression in the developing hypothalamus.](image-url)
Fgf3 regulates etv5b expression in the posterior hypothalamus

The transcription factor Etv5b is a downstream target of Fgf-signalling in various contexts (Mason, 2007; Ornitz and Itoh, 2015; Raible and Brand, 2001; Roehl and Nüsslein-Volhard, 2001; Roussigné and Blader, 2006). However, the identity of the Fgf(s) regulating etv5b and thereby the ontology of hypothalamic serotonergic populations remains unknown. Preliminary observations from Bosco et al. (2013) and our current fgf3 expression analysis (see above), suggest that Fgf3 might be the main Fgf-ligand in this context. To explore this hypothesis, we investigated hypothalamic etv5b expression in the fgf3 mutant, fgf3t24152, which was suggested to be a null mutant (amorph) (Herzog et al., 2004), as well as in fgf3 morphants. Our analysis revealed a reduced expression of etv5b in mutants compared to wild-type siblings at 36 hpf (Fig. 2A–C). Further, etv5b expression was reduced in a dose-dependent manner as the in situ hybridisation signal was weaker in homozygotes than in heterozygotes. Our RNA sequencing data supported these observations by revealing fewer etv5b transcripts in homozygous fgf3t24152 mutants than in wild types at 3 and 7 days post fertilisation (dpf) (Fig. 2F), although the read count values did not pass our defined thresholds. Similarly we observed a reduction of etv5b expression in fgf3 morphants compared to controls (Fig. 2D,E). However, the etv5b signal was never completely abolished, neither in our in situ hybridisation nor in our RNA sequencing experiments. Thus, we show that Fgf3 regulates etv5b expression in the developing posterior hypothalamus.

Fgf3 impacts on monoaminergic cell development in posterior hypothalamus

After identifying Fgf3 as a possible regulator of hypothalamic serotonergic CSF-c cell development based on expression data, we next tested this hypothesis functionally. For this, we used the fgf3t24152 mutant, which has a G to A transition point mutation resulting in a premature stop codon and, thereby, a truncated Fgf3 protein with 69.1% of the wild-type amino acid sequence remaining (Fig. S1D) (Herzog et al., 2004). Secondly, we created a fgf3 morpholino knockdown, which leads to a truncated protein containing 49.6% of the wild-type amino acid sequence (Fig. S1D). Finally, we applied CRISPR/Cas9 to generate indel mutations causing either a nonsense amino acid sequence or a premature stop close to the N-terminal (Fig. S2). All embryos with a manipulated fgf3, irrespective of the used strategy, showed similar defects in ear and craniofacial development at 72 hpf (Fig. S3). Embryos exhibited fused otoliths presumably due to a role of Fgf3 in anterior ear specification, and malformations of the ventral head skeleton attributed to the loss of ceratobranchial cartilage (Hammond and Whitfield, 2011; Herzog et al., 2004). Additionally, embryos with manipulated fgf3 displayed a defect in swim bladder development or inflation (not shown). The fact that we could confirm these phenotypes in homozygous fgf3t24152 mutants and reproduce similar defects in fgf3 morpholino and CRISPR/Cas9-injected embryos show that all three approaches to manipulate fgf3 produce qualitatively comparable results. Apart
from the described defects, no severe morphological abnormalities or increased overall cell death were observed (Figs S3, S4, S6). Next, we focused on the impact on hypothalamic serotonergic CSF-c cell development, applying all three strategies in parallel. The number of hypothalamic 5-HT immunoreactive cells in the intermediate (i.) and posterior (p.) populations were quantified at 72 hpf, a stage when the maturation of the serotonergic cells is well on the way (Bellipanni et al., 2002; Bosco et al., 2013; McLean and Fetcho, 2004). In addition, to label catecholaminergic cells, 3 dpf embryos were co-stained with a TH1 antibody, and 4 dpf embryos were processed for in situ hybridisation for th2 (Chen et al., 2009). TH1-positive cells of the posterior tuberculum/hypothalamus are subdivided into several subpopulations (Rink and Wullimann, 2002). We quantified the TH1 immunoreactive CSF-c cells in regions DC 4/5/6 and TH1 and th2 expressing cells in DC 7, which are dopaminergic (Filippi et al., 2009; Yamamoto et al., 2010, 2011, but see Xavier et al., 2017). Region DC 4/5/6 is located close to the p. serotonergic population (Kaslin and Panula, 2001; McLean and Fetcho, 2004).

We found that homozygous fgf3t24152 mutants, fgf3 morpholino and CRISPR/Cas9-injected embryos had reduced numbers of serotonergic CSF-c cells, but depending on the approach used the severity of the phenotype varied (Fig. 3). Specifically, homozygous fgf3t24152 mutants had 17% fewer serotonergic CSF-c cells than wild-type siblings (Fig. 3Q, Table S3), fgf3 morphants had a reduction of 45% compared to controls (Fig. 3T, Table S3), and CRISPR/Cas9-injected embryos had 49% fewer serotonergic CSF-c cells than uninjected controls and 42% fewer than control siblings injected with Cas9 only (Fig. 3W, Table S3). The reduction of i./p. serotonergic CSF-c cells persisted at 4 dpf in fgf3 morphants with a loss of 33% (Fig. S5, Table S3).

With respect to the TH1 immunoreactive cells, the numbers in DC 4/5/6 were never significantly affected after any of the fgf3 manipulations (Fig. 3R, U, X, Fig. S5, Table S3). In contrast, homozygous fgf3t24152 mutants, fgf3 morpholino and CRISPR/Cas9-injected embryos had fewer cells in DC 7 (Fig. 3). A reduction of TH1-positive cells by 32% was detectable in homozygous fgf3t24152 mutants compared to wild-type siblings (Fig. 3S, Table S3). Further, homozygous fgf3t24152 mutants had 38% fewer TH1-positive cells than heterozygotes. In fgf3 morphants, the number of TH1-expressing cells in DC 7 was reduced by 70% compared to uninjected controls (Fig. 3V, Table S3). CRISPR/Cas9-injected embryos had 35% fewer TH1-positive cells in comparison to uninjected controls, and 44% fewer compared to controls injected with Cas9 only (Fig. 3Y, Table S3). The reduction of TH1-positive DC 7 cells persisted at 4 dpf in fgf3 morphants with a loss of 34% (Fig. S5, Table S3). Similarly, the number of th2 expressing cells was reduced by 33% in morphants compared to controls (Fig. S5L–N, Table S3).

Taken together, using three independent techniques to manipulate fgf3 activity, we demonstrated a consistent loss of monoaminergic CSF-c cells after fgf3 impairment, showing a developmental dependency of these cells on Fgf3. Interestingly, this seemed to specifically affect the populations in the posterior hypothalamus.

**oxytocin (oxt) and cortistatin (cort) expressing neuroendocrine cells are unaffected by impaired fgf3 function**

To test the specific dependence of the monoaminergic CSF-c cells in the posterior hypothalamus on Fgf3, three additional cell populations, including neuroendocrine cells expressing *oxytocin (oxt)*, *arginine vasopressin (avp)* and *cortistatin (cort)* were investigated (Devis et al., 2002; Eaton et al., 2008; Unger and Glasgow, 2003). These populations were chosen due to their spatial proximity to the hypothalamic monoaminergic regions. fgf3t24152 mutants and fgf3 morphants were labelled for oxt, avp and cort at 72 hpf, and the number of positive cells was counted. The oxt and cort cell numbers were not decreased in hetero- and homozygous fgf3t24152 mutants or fgf3 morphants (Fig. 4, Table S3) compared to control siblings. However, there was a significant reduction in the number of avp-positive cells (Fig. 4E–H, Table S3). Homozygous fgf3t24152 mutants lost 16% of the cells compared to wild types and 14% compared to heterozygotes (Fig. 4O, Table S3). In fgf3 morphants, avp-expressing cell numbers decreased by 11% compared to controls (Fig. 4P, Table S3). Thus, avp-expressing, but not oxt- or cort-expressing cells showed dependency on Fgf3. Interestingly, the most posteriorly located avp-positive cells were more affected than anteriorly located cells in mutants and morphants (Fig. 4F, H), and therefore mirroring the effects on the TH1-positive cells.

**fgf3t24152 mutants and fgf3 morphants have a smaller hypothalamus**

To determine whether the loss of hypothalamic monoaminergic CSF-c cells, as well as avp-expressing cells consequently resulted in a smaller hypothalamus, we measured the size of the hypothalamic domain in fgf3t24152 mutants and fgf3 morphants. nkx2.4b expression at 36, 48 and 72 hpf was used to visualise the hypothalamic domain, which was subsequently measured in a semi-automatic manner (Fig. 5, Fig. S6). Qualitatively, neither the ventral nor the lateral silhouette of the nkx2.4b domain showed any major alterations after fgf3 impairment suggesting that the gross organisation of the domain is similar (Fig. 5, Fig. S6). However, we noticed a difference in size, which was more prominent from a ventral view compared to the lateral view. This notion can be explained by a developmental change in 3D shape of the domain going from a largely oval ‘egg’-shape at 36 hpf to a broader and flatter shape at 72 hpf, in particular at the lateral edges of the posterior end surrounding the posterior recess (Fig. S6K). The posterior hypothalamic end houses the prominent posterior serotonergic and DC7 dopaminergic populations, and a loss of those cells will consequently result in a smaller ventral silhouette. Concentrating on the ventral view, homozygous fgf3t24152 mutants showed a trend (6%) towards a smaller hypothalamic domain at 36 hpf (Fig. 5M, Table S3). At 48 hpf a significant reduction of 11% in homozygous fgf3t24152 mutants compared to wild-type siblings was recorded, and at 72 hpf the hypothalamus was smaller in homozygous fgf3t24152 mutants compared to both heterozygous mutant (10%) and wild-type (14%) siblings (Fig. 5O, Table S3). As expected, measurements of fgf3 morphants revealed significantly reduced hypothalamic size, but the body length was unchanged (Fig. 5, Fig. S6J). At 36 hpf the nkx2.4b-positive domain in fgf3 morphants was 19% smaller compared to uninjected controls (Fig. 5N, Fig. S6, Table S3). Further, at 48 and 72 hpf, the reduction was 16% and 15%, respectively (Fig. 5P, Table S3). Thus, we noticed that the size of the hypothalamic domain was more reduced in fgf3 morphants than in fgf3t24152 mutants at all stages examined, which was in line with the stronger monoaminergic phenotypes observed in fgf3 morphants compared to fgf3t24152 mutants described above.

**Fgf3 regulates proliferation and survival in the developing posterior hypothalamus**

fgf3 expression precedes the expression of *phila* and 5-HT by several hours (Bellipanni et al., 2002; Bosco et al., 2013). Further, in this study we noticed a reduction of hypothalamic size already, before the appearance of mature monoaminergic cells. Together these
Fig. 3. Quantification of the number of serotonergic cells in the intermediate (i.)/posterior (p.) clusters and of dopaminergic cells in the DC 4/5/6 and DC 7 clusters in the hypothalamus at 72 hpf after fgf3 impairment. (A–P) Confocal maximum intensity projections from wild-type controls (Ctr), homozygous fgf3t24152 mutants (−/−), fgf3 morphants (MO) or fgf3 CRISPR/Cas9-injected embryos (CR) immunostained for 5-HT (green) and TH1 (magenta) shown as single channels and merged. C,G,K and O show boxed areas in B,F,J and N, respectively, with adjusted brightness and contrast to reveal faint TH1 immunoreactive cells of the DC 7 cluster. Ventral views, anterior to the left. Scale bars: 10 µm. (Q–Y) Quantifications of 5-HT and TH1 positive cells after fgf3 impairment and in control siblings. The number of serotonergic cells was counted in the i./p. clusters as indicated by the line in A. The number of dopaminergic cells was counted in the DC 4/5/6 and DC 7 clusters as indicated by the lines in B and C. Tukey boxplots show median, 25–75% percentile, IQR whiskers and outliers. n=number of analysed individuals. +/-, heterozygous fgf3t24152 mutants; UC, uninjected siblings; C9C, injected with Cas9 only. *P>0.05, **P>0.01, ***P>0.001.
observations argue for an early effect of fgf3 on progenitors later giving rise to mature monoaminergic cells. To test for this possibility we analysed proliferation rate and cell death at 36 hpf in the posterior hypothalamus after impairment of fgf3 (Fig. 6, Table S3). Using 5-bromo-2′-deoxyuridine (BrdU) and phospho-histone H3 (phH3) we labelled cells in S-phase and M-phase, respectively. We noticed a significant reduction in the number of BrdU-positive cells, and a small, but not significant reduction in the number of phH3 immunoreactive cells (Fig. 6A–H), which together speaks for a lowered proliferation rate after fgf3 impairment. In parallel, we noticed an increased number of cleaved Caspase 3 (cCasp3) immunoreactive cells (Fig. 6I-K) showing elevated levels of cell death.

Fig. 4. Quantification of the number of oxt-, avp- and cort-expressing cells in the hypothalamus of fgf3 mutant and fgf3 morphants at 72 hpf. (A–L) Light microscopic pictures of wild type (+/+) and homozygous fgf3 mutant (−/−) siblings as well as fgf3 morphants (MO) and uninjected control siblings (UC) processed for RNA in situ hybridisation. The posterior cells expressing avp are more affected by impaired fgf3 than the anterior ones. Ventral views, anterior to the left. Scale bar: 30 µm. (M–R) Quantifications of oxt-, avp- and cort-positive cells in fgf3 mutant, fgf3 morphants and control siblings. Cell clusters used for the analyses are indicated by the lines in A, E and I. Tukey boxplots show median, 25–75% percentile, IQR whiskers and outliers. n=number of analysed individuals. +/−, heterozygous fgf3 mutants. *P>0.05, **P>0.01, ***P>0.001.
death. Control experiments of fgf3 morphants showed no significant alteration of the total body length (Fig. S6J), nor increased levels of cell death revealed by Acridine Orange and cCasp3 in morphants compared to control siblings (Fig. S4), showing that there is no general growth retardation or increased cell death due to loss of Fgf3 or morpholino injections.

\(\text{fgf3}^{t24152}\) mutant and morphant Fgf3 may still interact with Fgf receptors

To estimate the impact of the \(\text{fgf3}^{t24152}\) mutation and the exon2/intron2 splice blocking morpholino on the folding, stability and receptor binding capacity of the truncated isoforms of Fgf3 we carried out computational 3D modelling comparing both truncated
isomorphs to wild-type Fgf3 (Fig. 7). As described above, the \( \text{fgf3}^{24152} \) mutation resulted in an Fgf3 isoform with about 70% of the wild-type amino acid sequence remaining, while the splice morpholino generated an isoform with about 50% of the wild-type sequence intact (Fig. S1D). Notably, receptor binding is likely to still be possible for the \( \text{fgf3}^{24152} \) mutant Fgf3 since a major portion of the interfaces with the Fgf receptor may remain intact (Fig. 7B). As expected the 3D model of the morpholino knockdown isoform of Fgf3 showed less preserved structure than the \( \text{fgf3}^{24152} \) isoform and therefore a lower probability to interact with the receptor (Fig. 7C). Both isoforms are likely to be less stable than wild-type Fgf3.

\( \text{fgf3}^{24152} \) mutants exhibit minor alterations in the hypothalamic Fgf transcriptome

The paracrine family of Fgf ligands includes multiple members in addition to Fgf3 (Itoh, 2007). Further, Fgf-signalling contains numerous regulatory feedback systems (Ornitz and Itoh, 2015). Therefore, it may be that one or several previously overlooked Fgfs and/or that the feedback systems compensate for a loss of Fgf3 functionality. Such compensatory mechanisms could in turn explain why some of the posterior monoaminergic CSF-c and vasopressin-expressing cells remain after \( \text{fgf3} \) impairment. To test this possibility, we accomplished a transcriptome analysis.

RNA sequencing was performed on dissected hypothalami of \( \text{fgf3}^{t24152} \) mutants and wild-type cousins at 3 and 7 dpf. Of the 62 selected genes (Table S4) are expressed in the hypothalamus of all groups (Fig. 8B). In the wild-type groups, \( \text{fgfr}2, \text{fgfr}3 \) and \( \text{etv}1 \) and \( \text{etv}4 \) were upregulated in mutants at both 3 and 7 dpf compared to wild types of the same age (Fig. 8C), which indicates a self-compensatory mechanism of \( \text{fgf3} \). \( \text{fgf}11\text{b} \) and \( \text{fgf}24 \) were up- and downregulated, respectively, in mutants compared to wild types, but only at 7 dpf (Fig. 8C). All other detected \( \text{fgfs} \) did not pass our 1.5-fold change criteria for the mutant versus wild type comparisons. Moreover, our RNA sequencing data showed that all five \( \text{fgf} \) genes were expressed in the hypothalamus of all groups (Fig. 8B). In the wild-type groups, \( \text{fgfr}2, \text{fgfr}3 \) and \( \text{etv}4 \) were downregulated at 7 dpf compared to 3 dpf (Fig. 8C). Four out of four selected ETS-domain transcription factors, including \( \text{etv}1, \text{etv}4, \text{etv}5\text{a} \) and \( \text{etv}5\text{b} \), were expressed in the hypothalamus of all groups (Fig. 8B). Of these ETS-domain factors only \( \text{etv}5\text{a} \) expression increased beyond our predefined fold change threshold from 3 to 7 dpf in wild types, but did not change when comparing mutants to wild types at 3 or 7 dpf (Fig. 8C). RNA sequencing results of Fgf-signalling pathway genes revealed that 56 out of 62 selected genes (Table S4) are expressed in the hypothalamus of wild types and mutants at 3 and 7 dpf (Fig. 8D). None of these 56 genes were differentially expressed when comparing mutants to wild types at 3 or 7 dpf (Fig. 8B). Among these 56 genes were differentially expressed when comparing mutants and wild types at 3 dpf, but at 7 dpf, \( \text{dusp}1, \text{dusp}2 \) and \( \text{dusp}5 \) were downregulated in the mutants (Fig. 7E).

Next, we tested two Fgf-signalling target genes, which exhibited distinct expression profiles in the RNA sequencing analysis, by RNA in situ hybridisation at 36 hpf after morpholino knockdown, namely \( \text{dusp}1 \) and \( \text{dusp}6 \). In line with the RNA sequencing, \( \text{dusp}1 \) showed low expression levels in the hypothalamus and a mild reduction after \( \text{fgf3} \) impairment in the hypothalamus as well as elsewhere in the central nervous system. \( \text{dusp}6 \) expression was more prominent in the posterior hypothalamus, but did not reveal any obvious alterations in expression levels after \( \text{fgf3} \) impairment (Fig. 7E). Thus, the in situ hybridisation analyses showed similar
after fgf3 impairment before the appearance of mature serotonergic CSF-c cells, we propose that Fgf3 is critical during early stages when progenitors are still proliferating. This is supported by our observation that the number of proliferating cells is decreased in parallel with increased cell death after fgf3 impairment. This is in line with our earlier finding that Fgf-signalling, via etv5b, influences the proliferation of hypothalamic serotonergic CSF-c progenitor cells (Bosco et al., 2013). Furthermore, our present study provides evidence for the expression of previously overseen fgfs in the developing hypothalamus. We show that the expression of fgf3 is upregulated upon impairment of fgf3, suggesting activation of a self-compensatory mechanism. Together these findings highlight Fgf-signalling, in particular Fgf3, in a novel context as part of a signalling pathway of critical importance for hypothalamic development. Our results have implications for the understanding of vertebrate hypothalamic evolution.

**fgf3 is present in the posterior hypothalamus and likely acts as a morphogen regulating the expression of ETS-domain transcription factors**

Fgf-signalling, acting via Etv5b, is an important pathway for development of serotonergic CSF-c cells in the posterior hypothalamus (Bosco et al., 2013) as well as in other contexts (Mason, 2007; Ornitz and Itoh, 2015; Raible and Brand, 2001; Roehl and Nüsslein-Volhard, 2001; Roussigné and Blader, 2006). However, the Fgf ligands are many and their interactions with Fgfrs are promiscuous (Omitz and Itoh, 2015). To identify the Fgf ligand active in the developing posterior hypothalamus we searched existing expression data and found that fgf3 is present in its most posterior region (Herzog et al., 2004), an area that contains serotonergic CSF-c cells (Liilesaar, 2011; McLean and Fetcho, 2004). Thus, we hypothesised that Fgf3 is the main ligand responsible for Fgf activity in this particular region of the brain. As observed elsewhere (Herzog et al., 2004; Liu et al., 2013), we confirmed that fgf3 is expressed in the developing hypothalamus. Initially it has a broad distribution in the hypothalamic primordium. The expression then gradually becomes limited to the most posterior end in medially located cells surrounding the ventricle. Conforming to Fgf3 activity in the posterior hypothalamus, the Fgf responsive downstream targets etv4, etv5a and etv5b are present there, and largely overlap with fgf3 at early stages (Fig. 1P,U) (Bosco et al., 2013). However, at later stages and in contrast to fgf3 their expression is not limited to cells at the ventricle, but shows a broader distribution including cells in the parenchyme (Fig. 1Q,R,V) (Bosco et al., 2013), thus, fitting the role of Fgf3 as a morphogen (Bökel and Brand, 2013; Ornitz and Itoh, 2015). To test if Fgf3 regulates etv5b expression in the hypothalamus we analysed etv5b expression in fgf3t24152 mutants and fgf3 morphants. We noticed a reduction of etv5b transcripts in the posterior hypothalamus. These results demonstrate that Fgf3 is an important Fgf ligand in the developing posterior hypothalamus and that Fgf3 positively regulates etv5b expression in this brain region. Based on the distribution of transcripts it can be assumed that Fgf3 is secreted by a limited population of cells located at the ventricle, and reaches responsive cells expressing etv5b in more lateral positions. Based on our current findings we cannot conclude if fgf3 alone regulates etv5b and if the regulation is direct or indirect.

**Fgf3 activity is critical for monoaminergic CSF-c and avp-expressing cells in the posterior hypothalamus**

Hypothalamic serotonergic progenitors proliferate at around 36 hpf, and differentiated serotonergic CSF-c cells are detectable at 62 hpf
in the i/p. cluster (Bosco et al., 2013; McLean and Fetcho, 2004). Further, *etv5b* regulates the proliferation of serotonergic progenitors, and ultimately the number of mature serotonergic CSF-c cells (Bosco et al., 2013). These observations, in combination with our current finding that Fgf3 impacts on *etv5b* expression, prompted us to investigate whether impairment of *fgf3* would reduce the number of serotonergic CSF-c cells. By impairing *fgf3* we show that, indeed, serotonergic CSF-c cells depend on Fgf3.
Irrespective of the approach used, we always observed a reduction of hypothalamic serotonergic CSF-c cells. However, the population was never completely abolished, suggesting that some cells are independent of Fgf3, that our loss-of-function approaches result in only partial fgf3/Fgf3 impairment and/or that other Fgfs can partly compensate for the loss of fgf3/Fgf3 (for further details see below).

In the hypothalamus, dopaminergic CSF-c cells located in regions DC 4/5/6 and DC 7 are situated next to or intermingled with 5-HT immunoreactive cells of the i. and p. populations, respectively (Kaslin and Panula, 2001; McLean and Fetcho, 2004; Rink and Wullimann, 2002). Dopaminergic and serotonergic cells belong to the monoaminergic systems and accordingly share the expression of metabolic pathway genes, such as ddc, mao and slc18a2 (vmat2) (Yamamoto and Vernier, 2011). This may suggest a common developmental programme for hypothalamic monoaminergic populations, and therefore a mutual dependence on Fgf3. To test if the dopaminergic DC 4/5/6 and DC 7 cells require Fgf3, we quantified them after fgf3 impairment. Interestingly, we noticed a significant reduction only in the posteriorly located DC 7 population. Similarly, another study showed fewer dopaminergic cells specifically in the DC 7 population of fgf324152 mutants (Koch et al., 2014). In contrast, neither the DC 4/5/6 nor the DC 7 populations are affected in etv5b morphants (Bosco et al., 2013). To further expand the analysis of hypothalamic cell populations potentially affected by Fgf3 we investigated neuroendocrine cells expressing oct, avp and cort (Devos et al., 2002; Eaton et al., 2008; Unger and Glasow, 2003). Of these neuroendocrine populations, the oct- and cort-expressing ones were unaffected in fgf324152 and fgf3 morphants. Earlier quantifications of oct- and cort-expressing cells in etv5b morphants (Bosco et al., 2013) are similar to our present results after fgf3 impairment. Hence, we conclude that those two neuroendocrine populations are neither dependent on Etv5b nor Fgf3. In contrast, the number of avp-expressing cells was reduced in mutants and morphants suggesting that avp-expressing cells require Fgf3. Notably, the posterior avp-expressing cells were more affected than the anterior ones, similar to our observations of the dopaminergic DC 7 and DC 4/5/6 cells, thus supporting the hypothesis that Fgf3 plays a role predominantly in the posterior hypothalamus. Further, the avp-expressing population appears to be etv5b independent (Bosco et al., 2013). To summarise, the monoaminergic as well as avp-expressing cell populations require Fgf3. However, when comparing the different populations they exhibit distinct Fgf-signalling profiles; (1) etv5b is essential only for the posterior serotonergic cells, and (2) there is a posterior to anterior Fgf3 dependence gradient with the highest requirement seen in the posterior populations. We cannot exclude that etv5b-independent cell types use another ETS-domain transcription factor activated by Fgf3. The presence of etv5a and etv4 transcripts in this region (Bosco et al., 2013) renders such a scenario possible.

It has been proposed that species-specific developmental programmes may result in ‘hypothalamic modules’, which can be gained or lost during evolution (Xie and Dorsky, 2017). One striking example of such a putative module is the posterior paraventricular organ surrounding the posterior recess, which houses the monoaminergic CSF-c cells. This particular structure is found in teleosts, but absent in tetrapods (Xavier et al., 2017). Thus, it is plausible that Fgf3 and Etv5b are part of a developmental signalling programme promoting the formation of a posterior hypothalamic module.

**Fgf3 likely regulates proliferation of monoaminergic progenitors**

The monoaminergic CSF-c cells constitute a considerably large population of cells in the posterior hypothalamus. We therefore hypothesised that loss of those cells, as seen after fgf3 impairment, would be associated with a smaller hypothalamus. As expected, we observed a reduction of the nkh2.4b-positive domain at 72 hpf, a stage when numerous monoaminergic cells are present. Interestingly, the size reduction was observable at stages before the appearance of mature monoaminergic cells. This suggests that Fgf3 activity plays a role prior to complete cell maturation, presumably at a stage when monoaminergic progenitors are still proliferating, which is in line with the proposed function of Etv5b (Bosco et al., 2013). Accordingly, we here showed that the number of proliferating cells is reduced in the posterior hypothalamus at 36 hpf after fgf3 impairment. Further, we found that the number of cCas3p3 immunoreactive cells was increased in the posterior hypothalamus. Notably, we did not observe any overall morphological signs of increased cell death or growth retardation in fgf3-impaired embryos (mutants, morphants or CRISPR/Cas9) at any stage investigated, and no general increase of cell death in fgf3 morphants speaking against a generally increased cell death or developmental retardation. Taken together this demonstrates that fgf3 is required for the regulation of proliferation of progenitors in the posterior hypothalamus. The increased levels of hypothalamic cell death may be explained by Fgf3 acting as a survival factor, and/or it may be a secondary consequence of progenitors failing to follow their normal cell cycle programme. With our current experiments we cannot rule out that Fgf3 may also impact on cell fate and differentiation. We conclude that the change in hypothalamic size as revealed by the size of the nkh2.4b domain is caused by two temporally distinct processes, first the early reduced proliferation and increased cell death, and second the failure to generate mature monoaminergic cells populating the posterior hypothalamus.

**The fgf324152 mutant exhibits a milder phenotype than fgf3 morphants or fgf3 CRISPR/Cas9-injected embryos**

All three strategies (mutant, morpholino and CRISPR/Cas9) to interrupt fgf3 function resulted in a reduction of monoaminergic CSF-c cells, with up to about 50% loss of serotonergic CSF-c cells on average in fgf3 CRISPR/Cas9-injected embryos. However, we never saw a complete loss of these cells. This may be explained by: (1) some wild-type Fgf3 remaining after manipulation, (2) that the fgf324152 allele is not a morph, hence, not leading to a complete loss
of Fgf3 activity, (3) compensatory mechanisms (upregulation of fgf3 itself, upregulation of other fgfs, alterations in the downstream Fgf-signalling pathway and/or in its feedback regulators) and/or (4) that some serotonergic cells develop independently of Fgf3.

Our 3D models of the proteins resulting from the fgf3^{24152} mutation and the morpholino knockdown suggest that both isoforms are less stable than the wild-type protein, with the morphant protein being the least stable. However, according to the 3D models showing the ligand/receptor complex, both truncated isoforms may still interact with FgfRs (Fig. 7). Again, the morphant form exhibits the more severe alteration. In the morphant, wild-type transcripts are detectable, and it is therefore likely that we do not have a complete loss-of-function. The fgf3^{24152} allele was published as being a nonsense mutation likely to be amorphic leading to a truncated Fgf3 protein with a complete loss of activity (Herzog et al., 2004). Considering this, we expected the homozygous fgf3^{24152} mutant to exhibit a more severe hypothalamic phenotype than we observed. With the 3D models and our phenotypic characterisation of the fgf3^{24152} mutant at hand we question the previous conclusion that the fgf3^{24152} mutation is amorphic, and propose that the fgf3^{24152} allele rather corresponds to a hypomorphic mutation.

Our CRISPR/Cas9 experiments were performed in the F0 generation and the mutations will therefore consist of various indels and have a mosaic distribution. The target for our exon 1 guide RNA is situated 153 base pairs from the start codon. In the most severe indel mutation scenario we therefore expect to lose about 80% of the wild-type amino acid sequence. As can be expected in F0-injected embryos, we saw a more variable strength of the phenotype between individuals compared to fgf3^{24152} mutants or morphants. In some individuals we noticed an almost complete loss of the posterior monoaminergic cell populations, which correlated with a strongly reduced posterior hypothalamus (Fig. 3M–P). Although we cannot finally conclude from our current data, it is likely that such individuals have lost most, if not all, functional Fgfr3. In future studies of stable fgf3 CRISPR lines it will be interesting to see if there is a complete loss of the posterior hypothalamic CSF-c cells.

Our RNA sequencing results showed that several fgf genes are expressed in the hypothalamus making them possible candidates for compensatory mechanisms. Of these fgfs the only one being upregulated in homozygous fgf3^{24152} mutants and passing the defined fold change threshold both at 3 and 7 dpf was fgf3 itself, arguing for a self-compensatory role. Further, fgf11b was upregulated in mutants, but only at 7 dpf. fgf8a also showed a mild upregulation, but did not pass the defined threshold. fgf8a transcripts have been detected by in situ hybridisation in the posterior hypothalamus, but are spatially more restricted compared to fgf3 (Reifers et al., 1998). The differential expression of downstream Fgf-signalling and feedback regulator genes was moderate in the fgf3^{24152} mutants, but a few of them were downregulated at 7 dpf. Among these were three dusp genes (dusp1, 2 and 5), which act as negative feedback regulators of mitogen-activated protein kinases (Cant and Keyse, 2013; Ornitz and Itoh, 2015; Zniosko et al., 2010). Also dusp6 showed a mild downregulation, but did not fulfil our selection criteria. In situ hybridisation for dusp1 and dusp6 after fgf3 impairment by morpholinos support these findings. Taken together, these results indicate that a loss of Fgf3 activity in the fgf3^{24152} mutant is compensated for, but only mildly, on multiple levels by both self-compensation and by other Fgf ligands, as well as by alterations in the downstream signalling.

In addition to Fgf-signalling, other signalling pathways and gene regulatory networks are active in the zebrafish developing posterior hypothalamus. For instance, lef1, a direct mediator of Wnt-signalling, is transcribed there, and is required for the expression of proneural and neuronal genes (Lee et al., 2006). A likely Wnt candidate in this context is Wnt8b (Lee et al., 2006). Supporting a role for Wnt-signalling in hypothalamic serotonergic neurogenesis, a subset of 5-HT immuno-reactive hypothalamic cells express Wnt-activity reporters (Lee et al., 2006; Wang et al., 2009, 2012). Further, functional studies have shown that hypothalamic Wnt-responsive cells are immature cells contributing to GABAergic and serotonergic populations (Wang et al., 2012). Impairment of fezf2 in zebrafish results in fewer serotonergic, dopaminergic and oxytocinergic cells (Blechman et al., 2007; Guo et al., 1999; Jeong et al., 2006; Levkovitz et al., 2003; Rink and Guo, 2004), and at least for the dopaminergic populations this seems to involve a Fezf2-dependant regulation of the transcription factors Neurogenin 1 and Orthopedia (Jeong et al., 2006; Shimizu and Hibi, 2009; Blechman et al., 2007; Ryu et al., 2007). If, and to which extent, these signalling pathways interact with Fgf-signalling to promote the generation of monoaminergic CSF-c cells remains a subject of investigation.
using the GenElute Gel Extraction Kit (Sigma-Aldrich) and verified by Sanger sequencing (Eurofins).

For the fgf3 CRISPR/Cas9 strategy two different gRNAs targeting exon 1 and exon 2 of fgf3 (Fig. S2) were designed using the web tool CHOPCHOP (Labun et al., 2016; Montague et al., 2014). gRNA oligos (Table S1) were annealed, cloned into vector DR274 (a kind gift from Keith Joung Addgene plasmid #42250), in vitro transcribed using T7 RNA polymerase (a kind gift from Thomas Ziegenhals and Utz Fisher) and purified by Roti-Aqua-phenol/chloroform/isomylalcohol (Roth) extraction. AB/AB embryos were co-injected at the one-cell stage with a cocktail containing both gRNAs (100–125 ng/µl each) and Cas9-NLS protein (300 ng/µl, S. pyogenes, New England Biolabs). To verify that the gRNAs induced indel mutations at the expected sites of fgf3, the target sites were amplified by PCR using fwd and rev primers listed in Table S1 followed by separation of the PCR products on a 3% high-resolution NuSieve 3:1 agarose gel (Lonza), PCR clean up using the GenElute PCR Clean-up Kit (Sigma-Aldrich) and Sanger sequencing (Eurofins).

For both, morpholino and CRISPR/Cas9, strategies uninjected stage matched siblings were used as controls. Additionally, Cas9-NLS protein only injected embryos were used as injection controls for the CRISPR/Cas9 experiments.

**Morpholino toxicity assay using Acridine Orange**

Live 24 hpf fgf3 morpholino-injected embryos and uninjected wild-type controls were incubated in 5 µl/ml Acridine Orange dissolved in Danieu’s solution for 30 min at 28°C, rinsed and imaged to reveal cell death.

**Brdu labelling**

fgf3 morpholino-injected and wild-type control siblings were pulsed with 10 nM 5-bromo-2′-deoxyuridine (Brdu, Sigma-Aldrich) in 15% DMSO in Danieu’s solution at 36 hpf for 20 min on ice, rinsed and directly fixed.

**Immunohistochemistry**

Brdu and phH3 whole-mount immunohistochemistry was performed on embryos at 36 hpf. After rehydration embryos were pre-treated with 10 µg/ml protease K for 20 min, followed by antigen retrieval in 2 N HCl for 1 h and then in 50% methanol in PBT for 1 h at −20°C followed by a quick rinse in H2O. Before labelling with Brdu and phH3 or ccasp3 antibodies the embryos were incubated in Brdu immuno blocking buffer [10% normal sheep serum, 0.2% bovine serum albumin in PBS with 0.1% Tween 20 (PBT) and 1% DMSO] for 1 h at room temperature. 5-HT and TH immunohistochemistry was performed on embryos with heads dissected free of skin, eyes, and jaws for better antibody penetration. Dissected embryos were incubated in immunoblocking buffer (10% normal sheep serum, 0.2% bovine serum albumin in PBT) for 2 h at room temperature. Embryos were subsequently labelled with rat anti-Brdu (1:200; BU1-75, antibodies-online), rabbit anti-phH3 (1:300; 06-570, Millipore), rabbit anti-cCasp3 (1:500; 9664, Cell Signalling), mouse anti-TH (1:500; MAB318, Millipore) and rabbit anti-5-HT (1:1000; SS545, Sigma-Aldrich) diluted in respective blocking buffers for 3 days at 4°C with gentle shaking. After washes in PBT, to reveal immunoreactivity, the specimens were incubated for 2 days at 4°C in secondary antibodies conjugated with either Alexa Fluor 488 (goat anti-rabbit, 1:1000; A-11034, Thermo Fisher Scientific) and/or Alexa Fluor 568 (donkey anti-mouse, 1:1000; A-10037, Thermo Fisher Scientific) diluted in respective blocking buffer.

**Whole-mount RNA in situ hybridisation**

To detect mRNA transcripts whole-mount in situ hybridisation was performed on fixed embryos as described elsewhere (Thiisse and Thiisse, 2008). In brief, in vitro transcribed digoxigenin (DIG) or fluoroescein (fluor) labelled antisense RNA probes (Table S2) were synthesised using linearised plasmid template DNA, suitable RNA polymerases and DIG or fluo RNA labelling mix (Roche) following manufacturer’s recommendations. Prehybridisation, hybridisation and stringency washes were performed at 65°C. Alkaline phosphatase conjugated to anti-DIG (1:5000, Roche) or anti-fluo (1:2000, Roche) Fab fragments were used to label hybridised transcripts, and to enable subsequent colour precipitation with NBT/BCIP solution (Roche) or Fast Red tablets (Sigma-Aldrich) dissolved in 0.1 M Tris-HCl with 0.1% Tween 20. For double in situ hybridisation, embryos were first labelled with anti-fluo antibody, which was revealed using Fast Red followed by heat detachment at 68°C for 2 h in PBT, followed by anti-DIG antibody labelling and NBT/BCIP revealing.

**Cryosections**

For histology, embryos were cryo sectioned after in situ hybridisation. Specimens were cryoprotected in 15% sucrose in PBS overnight, followed by embedding in 7.5% porcine skin gelatine (300 Bloom, Sigma-Aldrich) 15% sucrose solution in PBS. Gelatine blocks were cut and snap frozen in 2-methylbutane cooled down in a liquid nitrogen and were then stored at −80°C. 20 µm thick frontal crossections were cut on a cryostat (Microm HM 500 OM), collected on SuperFrost Plus slides (Thermo Fisher Scientific), rinsed in PBS and coverslipped.

**Sample preparation and data analysis of RNA sequencing**

Hypothalami of 3 and 7 dpf homozygous wild-type embryos (Tü/Tü) and fgf3T24152 homozygous mutant embryos (in Tü/Tü background) were dissected (Fig. 8A). Wild-type embryos were generated by crossing previously identified homozygous wild-type adult fish, which were siblings to the heterozygous parents used to generate fgf3T24152 homozygous mutant embryos. Thus, the homozygous wild type and the fgf3T24152 homozygous mutant embryos used for the RNA sequencing analysis were cousins. The fgf3T24152 homozygous mutant embryos were identified by their characteristic fused otolith phenotype (Herzog et al., 2004). Dissections were performed on ice in a petri dish containing ice cold slicing solution (234 mM sucrose, 11 mM D-glucose, 2.5 mM KCl, 1.25 mM NaH2PO4, 0.5 mM CaCl2, 2.0 mM MgSO4, 26 mM NaHCO3) (Ma et al., 2015) using forceps. To collect sufficient material for RNA sequencing (∼100 ng total RNA) hypothalami were pooled. For each of the four groups (3 and 7 dpf wild-type and mutant embryos) three independent replicates were collected adding up to a total of 12 samples. The collected tissue was preserved in RNAlater RNA stabilisation solution (Qiagen) until RNA was isolated using RNeasy Mini Kit (Qiagen) according to manufacturer’s instructions. RNA Library preparation was performed by the Core Unit Systems Medicine of the University of Würzburg according to the Illumina TrueSeq stranded mRNA Sample Preparation Guide with 100 ng of input RNA and 15 PCR cycles. All 12 libraries were pooled and sequenced on a NextSeq 500 with a read length of 150 nt. Sequenced reads were mapped with the RNA-Seq aligner software STAR (Dobin et al., 2013) to the Ensembl Danio rerio genome version GRCz10. Expected read counts were calculated by RSEM (Li and Dewey, 2011). For detection of differentially expressed genes the Bioconductor/R package DESeq2 was used (Love et al., 2014). gRNA oligos (Table S1) were first labelled with anti-fluo antibody, which was revealed using Fast Red followed by heat detachment at 68°C for 2 h in PBT, followed by anti-DIG antibody labelling and NBT/BCIP revealing.

**3D structural analysis**

For 3D modelling of the zebrafish Fgf3 structure the amino acid sequence was sent to the Phyre2 server (Kelley et al., 2015). 155 residues from the entire sequence were modelled with a 100% confidence level using pdb entry 1hhk (Piotnikov et al., 2001) and were subsequently used for the structural assessment of the Fgf3 variants. The structure of Fgf3 wild-type protein as well as truncated Fgf3 mutant (fgf3T24152) and morphant protein were modelled bound to a human FGF1 receptor based on the pdb entry 3ojv (Beenken et al., 2012). The structures were visualised with PyMOL
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Data availability
RNA-seq data have been deposited at the National Center for Biotechnology Information (NCBI) with accession number PRJNA541414.

Supplementary information
Supplementary information available online at http://bio.biologists.org/lookup/doi/10.1242/bio.040683.supplemental

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