Development of hypothalamic serotoninergic neurons requires Fgf signalling via the ETS-domain transcription factor Etv5b

Adriana Bosco1, Charlotte Bureau2, Pierre Affaticati2, Patricia Gaspar3,4, Laure Bally-Cuif1 and Christina Lillesaar1,∗

SUMMARY
Serotonin is a monoamine neurotransmitter that is involved in numerous physiological functions and its dysregulation is implicated in various psychiatric diseases. In all non-placental vertebrates, serotoninergic (5-HT) neurons are present in several regions of the brain, including the hypothalamus. In placental mammals, however, 5-HT neurons are located in the raphe nuclei only. In all species, though, 5-HT neurons constitute a functionally and molecularly heterogeneous population. How the non-raphe 5-HT populations are developmentally encoded is unknown. Using the zebrafish model we show that, in contrast to the raphe populations, hypothalamic 5-HT neurons are generated independently of the ETS-domain transcription factor Pet1 (Fev). By applying a combination of pharmacological tools and gene knockdown and/or overexpression experiments, we demonstrate that Fgf signalling acts via another ETS-domain transcription factor, Etv5b (Erm), to induce hypothalamic 5-HT neurons. We provide evidence that Etv5b exerts its effects by regulating cell cycle parameters in 5-HT progenitors. Our results highlight a novel role for Etv5b in neuronal development and provide support for the existence of a developmental heterogeneity among 5-HT neurons in their requirement for ETS-domain transcription factors.

KEY WORDS: Etv5b (Erm), Serotonin (5-hydroxytryptamine), Fgf signalling, Zebrafish

INTRODUCTION
The serotoninergic (5-HT) neurons of the vertebrate CNS represent a heterogeneous group with respect to metabolic pathways, anatomical characteristics, axonal morphology, neurochemistry, neurotransmission and electrophysiological properties (Gaspar and Lillesaar, 2012). This diversity is likely to be reflected in a molecular and developmental heterogeneity.

Representatives from all non-placental vertebrate taxa, including zebrafish, have serotonin-containing/producing neurons located in the pretectum, the basal forebrain (hypothalamus and/or posterior tuberculum), the hindbrain raphe nuclei and in the spinal cord (Lillesaar, 2011). Hence, the placental mammalian CNS, where 5-HT neurons are found exclusively in the raphe nuclei (Dahlström and Fuxe, 1964; Jacobs and Azmitia, 1992), represents an exception. 5-HT neurons are identifiable by a set of 5-HT identity proteins, including Aromatic amino acid decarboxylase, Tryptophan hydroxylase (Tph), Serotonin transporter, Vesicular monoamine transporter 2 and serotonin autoreceptors (Flames and Hobert, 2011), which are directly regulated by the ETS-domain transcription factor Pet1 (also known as Pet-1 or Fev) (Hendricks et al., 1999). In zebrafish, the various 5-HT populations express different paralogues of the 5-HT identity genes (Lillesaar, 2011; Norton et al., 2008).

Fgf and Shh signalling, acting via transcriptional networks, regulate the generation of raphe 5-HT neurons (Cheng et al., 2007; Kiyasova and Gaspar, 2011; Norton et al., 2005). Little is known about signalling molecules and transcriptional networks involved in the development of the non-raphe 5-HT populations. Within the developing zebrafish forebrain, local sources of Wnt, Hh, Nodal and Fgf are present (Machluf et al., 2011; Walshe and Mason, 2003). Accordingly, a molecular reporter for Wnt signalling is expressed in some hypothalamic 5-HT cells (Wang et al., 2009). Further, a zebrafish smoothened mutant exhibited a reduction of 5-HT cells in basal forebrain and raphe populations, whereas an shh mutation (syn) exclusively affected the raphe nuclei (Teraoka et al., 2004). Treatment of zebrafish embryos with the Fgf signalling inhibitor SU5402 reduced the number of 5-HT cells in raphe and basal forebrain, whereas in the zebrafish fgf8a mutant (ace) only the raphe population was affected. Together, these observations indicate the action of Hedgehog factors and Fgf other than Fgf8a and Shh in the basal forebrain 5-HT populations. Finally, the transcription elongation factor Foggy (Supt5h) was shown to control the ratio of dopaminergic (DA) to 5-HT neurons in the zebrafish hypothalamus (Guo et al., 2000), and the formation of these neurons depends on the zinc-finger transcription factor Fezf2/Tof in a non-autonomous manner (Levkowitz et al., 2003; Rink and Guo, 2004). However, a comprehensive exploration of 5-HT neuron development in the basal forebrain is missing.

Pet1 is pivotal for the maturation of raphe 5-HT neurons by controlling expression of the 5-HT identity genes (Hendricks et al., 1999; Liu et al., 2010). Interestingly, pet1 is only expressed in hindbrain 5-HT neurons in zebrafish (Lillesaar et al., 2007) and no paralogous gene, which could indicate subfunctionalisation, has been found (Zv9, www.ensembl.org/Danio_rerio/). We reasoned that some molecular similarities should exist in the cascades driving 5-HT development in the different brain subdivisions, and
we therefore searched for Fgf-dependent ETS-domain transcription factors expressed in hypothalamic 5-HT neurons or their precursors. In zebrafish, four members of the Polyomavirus enhancer activator 3 (Pea3) subfamily of ETS-domain transcription factors have been identified; pea3 (etv4), er81 (etv1) and two paralogues of etv5 designated etv5a (etv5) and etv5b (erm) (Brown et al., 1998; Münchberg et al., 1999; Roussigné and Blader, 2006). The expression of these genes, except er81, is controlled by the Fgf-induced MAPK pathway (Roussigné and Blader, 2006).

Here, we demonstrate that etv5b is expressed in precursors of 5-HT neurons of the hypothalamus and mediates Fgf signalling during their generation. We show that Etv5b does not impact on raphe 5-HT neurons. Furthermore, our findings show that Etv5b controls the final number of hypothalamic 5-HT cells by enabling cell cycle progression in proliferating progenitors. Our investigation allows, for the first time, a model to be proposed for the generation of hypothalamic 5-HT neurons in a vertebrate, and provides support for the existence of a developmental heterogeneity among 5-HT neurons in their requirement for ETS-domain transcription factors.

**MATERIALS AND METHODS**

**Fish strains**

Wild-type zebrafish (AB or EKk strains) were used. The Tg(etv5b:gf) transgenic line expressing Gal4:VP16 under the control of etv5b regulatory elements (Esain et al., 2010) (kindly provided by Julien Ghislain) was intercrossed with the Tg(tuas:gf) (Scheer et al., 2001) line used for lineage-tracing experiments. For cell cycle experiments, embryos from an intercross of the Tg(EF1α:mo22-Cdt1) and Tg(EF1α:AG-hGem) lines (Sakaue-Sawano et al., 2008) based on the Fucci technique (Sugiyama et al., 2009) were used. The Notch reporter line Tg(Tp1bglob:eGFP) (Parsons et al., 2009) was used for testing the influence of Fgf signalling on the Notch pathway. Embryos were maintained and staged as described (Kimmel et al., 1995).

**Injections**

Loss- or gain-of-function experiments were performed by morpholino (MO) or capped RNA injections into one-cell stage eggs. MOs (all from Gene Tools) targeting the start codons of etv5b (5'-AACCCATCATGTGCGCTTCTTC-3'), etv5a (5'-ATCCGTCATGTCACTGGGTT-3'), pea3 (5'-ATCCATGCTTTAACGGTGG-3') or pea3 (5'-ATCCATGCTTTAACGGTGG-3') were injected at 0.05-0.25 mM, at which concentrations no non-specific developmental defects were observed. A second MO targeting the splice donor of intron 1 of etv5b (0.15 mM; 5'-GCCTCTTAACACCTAGCTACC-3') was used to confirm the phenotype observed in etv5b Atg morphants (Mao et al., 2009). A 5 bp mismatch MO served as a negative control (0.15 mM; 5'-GGTTCTTTTAAAGACTCACCACCTGCTC-3') (Mao et al., 2009). The efficiency of the etv5b splice MO was evaluated by RT-PCR (Mao et al., 2009). Further, etv5a and pea3 ATG and etv5b splice MOs were injected in all possible double and triple combinations. In the triple combination, pea3 and etv5b MOs were reduced to 0.1 mM. For generation of etv5b RNA, full-length etv5b was PCR amplified (forward primer, 5'-CTTCGATGGTGGGGTTTATGACCAGC-3'; reverse primer, 5'-GATACTCAGAAAGTTGTAACCTCAGAAGG-3') from a pBluescript SK- vector containing the gene (RZPD clone IRAKp961E20136Q2), cloned into the XhoI/EcoRV sites of pXT7 (Dominguez et al., 1995) and verified by sequencing. Capped RNA was synthesised using the T7 mMessage mMachine Kit (Ambion) following the supplier’s instructions after linearisation with Smal. RNA was injected at 25, 50, 100 or 200 ng/jl. Rescue experiments were performed by co-injecting etv5b splice MO (0.15 mM) with etv5b RNA (50 ng/jl).

**SU5402 treatment**

Embryos were dechorionated and incubated in embryo medium (E3) containing 10 µM SU5402 (Calbiochem/Merck) dissolved in DMSO (0.1% final concentration) or in 0.1% DMSO only (control) during the indicated stages. After treatment, embryos were washed twice and transferred to fresh plates. Epistasis experiments were performed by injecting etv5b capped RNA (50 ng/jl) at the one-cell stage followed by SU5402 treatment (10 µM) during 24-36 hours post-fertilisation (hpf) and fixation at 72 hpf.

**BrdU labelling**

Dechorionated wild-type embryos were soaked for 20 minutes on ice in 10 mM BrdU (Sigma-Aldrich) in 15% DMSO in E3 medium at 36 or 45 hpf, followed by three washes with E3 on ice, left for 20 minutes at 28.5°C, and then fixed immediately or at 72 hpf. To examine when, during embryonic development, the majority of the hypothalamic 5-HT precursor cells are proliferating, a birthdating experiment was performed by exposing embryos to BrdU as described above starting at 36, 42, 48 or 54 hpf, and then repeatedly twice per day until 54 hpf. Treated embryos were fixed at 72 hpf and processed for double BrdU/5-HT immunohistochemistry.

**In situ hybridisation and immunohistochemistry**

For primary antibodies and *in situ* hybridisation (ISH) probes, see supplementary material Table S1. To generate a gfp probe, gfp was PCR amplified (forward primer, 5'-ACGTAACGCCCACAGTTCTC-3'; reverse primer, 5'-CTTGTACAGCTGCTCAGG-3') and cloned into PCRII (Invitrogen). Whole-mount ISH or immunohistochemistry was performed on 4% paraformaldehyde (PFA)-fixed embryos (Lillesaar et al., 2009; Thiese et al., 1993). For double-fluorescent ISH, digoxigenin-labelled and fluorescein-labelled probes were detected using antidigoxigenin/fluorescein-POD Fab fragments (1/300, Roche) and revealed (Davidson and Keller, 1999) using FITC (1/100, PerkinElmer) or TAMRA (1/100, Invitrogen) conjugated tyramide.

For histological sections, embryos were fixed in 4% PFA, cryoprotected in 15% sucrose, embedded in gelatin:sucrose (7.5%;15% in PBS) and cryosectioned (15 μm). Primary antibodies were revealed using Alexa Fluor-conjugated goat secondary antibodies (488 or 546; 1/1000, Invitrogen). Sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI, 1/10,000) and mounted in AquaPolymount (Polysciences). For 5-HT and Tyrosine hydroxylase 1 (TH1; Th – Zebrafish Information Network) whole-mount immunohistochemistry, primary antibodies were detected with goat secondary antibodies coupled to Alexa Fluor dyes or biotin (1/200, Jackson ImmunoResearch) and revealed with streptavidin–horseradish peroxidase and SigmaFAST 3,3'-diaminobenzidine (DAB, Sigma–Aldrich). BrdU immunohistochemistry involved pretreatment with 3.3 M HCl for 30 minutes at room temperature. MO22 and mAG immunohistochemistry was performed according to the manufacturer’s protocol (Medical and Biological Laboratories).

**Image acquisition, quantification and statistics**

Cells were quantified and photographed using a DM5000B microscope (Leica Microsystems) equipped with a DFC420C digital camera and processed with Leica Application Suite (version 3.7.0). Fluorescent stainings were further documented as z-stacks with an LSM700 confocal microscope and ZEN 2009 software (Carl Zeiss MicroImaging). Subsequent image processing was performed using ZEN 2009 Light Edition (Carl Zeiss), Photoshop CS4 (version 11.0) and Illustrator CS4 (version 14.0.0) (Adobe Systems). Quantifications on cryosections were made from four to eight sections per embryo in each of the channels (405, 488 and 543 nm) on single optical sections (~1 μm), and two optical sections per z-stack were included in the analysis. Single optical sections or maximum intensity projections are shown in the figures.

Cell numbers are expressed as a percentage of the mean value calculated from un.injected/unreated control siblings and are given as mean ± s.e.m. For statistical analysis Student’s *t*-test was applied, and *P*≤0.05 was considered significant.

**RESULTS**

**Embryonic hypothalamic 5-HT precursor cells express the ETS-domain transcription factor Etv5b**

The 5-HT population in the basal forebrain can be subdivided into three distinct clusters: one medially located anterior group (a.) at the hypothalamus/posterior tuberculum border; a large cluster in...
the caudal part of the hypothalamus (p.); and an intermediate cluster (i.) (Kaslin and Panula, 2001). The developmental maturation of these groups follows an anterior-to-posterior gradient, with cluster a. expressing tph1a and 5-HT before 48 hpf, whereas the first 5-HT cells do not appear until ~60 hpf in clusters i./p. (Fig. 1A-C) (Bellipanni et al., 2002; Lillesaar et al., 2007; McLean and Fetcho, 2004).

We screened a zebrafish expression database (www.zfin.org) for ETS-domain transcription factors expressed in the developing basal forebrain. Our search revealed three members of the pea3 family as candidates: pea3, etv5a and etv5b. In the basal forebrain, etv5b exhibited the most prominent expression (Fig. 1D-G). At 24 hpf, etv5b was detected in the ventral forebrain, then progressively the expression narrowed, and by 30 and 36 hpf it was present in the ventral/caudal hypothalamus and in a part of the posterior tuberculum/hypothalamus. Expression persisted in this region until 48 hpf, when transcripts were found in an anterior-dorsal domain of the posterior tuberculum/hypothalamus where cluster a. is located, in addition to a ventral-caudal domain of hypothalamus that later overlaps with clusters i./p. etv5a exhibited a similar spatiotemporal pattern, albeit at lower levels (supplementary material Fig. S1A-D), whereas hypothalamic pea3 expression was more restricted (supplementary material Fig. S1E-H).

We focused on the hypothalamic (i./p.) 5-HT clusters and analysed whether these neurons originate from cells expressing etv5b. etv5b expression is strongest before 5-HT is detectable, and as we were unsuccessful at performing 5-HT immunohistochemistry after ISH, we took advantage of the ermp:gv and uas:gfp transgenic lines (Esain et al., 2010; Scheer et al., 2001). Transcripts for gfp and etv5b co-localised at 30, 36 and 40 hpf in the hypothalamus (Fig. 1H; confocal maximum intensity projection of a Tg(erm:gv)×Tg(uas:gfp) 30-hpf embryo processed for double gfp/etv5b ISH. Lateral view, anterior left. I-K) High magnifications of boxed area in H; confocal optical sections. (L-N) Confocal optical section of brain from a Tg(erm:gv)×Tg(uas:gfp) larvae revealing co-expression of GFP and 5-HT in neurons of the i./p. clusters. Ventral view, anterior left. White arrowheads indicate double-positive cells; open arrowheads indicate cells that are 5-HT-positive only. Scale bars: 50 μm.

**Etv5b is required for the development of hypothalamic 5-HT neurons**

To test whether Etv5b is involved in the development of hypothalamic 5-HT neurons, an MO targeting the start codon of etv5b (Znosko et al., 2010) was injected into wild-type eggs. Although no morphological or positional differences were observed
among the hypothalamic 5-HT neurons in morphants (Fig. 2A,B), the number of 5-HT-positive neurons in clusters i./p. was reduced (89.8±2.8%) compared with uninjected control siblings (100.0±2.8%; \( P = 0.011 \)) (Fig. 2U). The number of 5-HT cells in the anterior raphe (r. population) was unchanged (99.2±1.8% of uninjected control siblings; \( P = 0.755 \)) (Fig. 2C,D,U). This phenotype occurred in the absence of anomalies in the growth or morphology of the hypothalamic region, as assessed by normal expression of nkx2.1a (Fig. 2K-N). Moreover, apoptosis levels revealed by immunohistochemistry for cleaved caspase 3 on cryosections were unchanged in morphants at 36 hpf (data not shown).

The observed phenotype was confirmed with a second MO targeting etv5b splicing (Mao et al., 2009). The efficiency of the splice MO was tested by RT-PCR. Injected embryos exhibited an almost complete loss of wild-type RNA (supplementary material Fig. S2A). The etv5b splice MO resulted in a reduction in the number of 5-HT-positive neurons (84.6±2.5% of uninjected control siblings; \( P=7\times10^{-6} \)) (Fig. 2E-G,V). A mismatch control MO gave a phenotype comparable to that of uninjected control siblings (94.3±2.2%; \( P = 0.064 \)). The r. population was unchanged after injection of either the splice (102.0±1.4%; \( P = 0.343 \)) or mismatch (102.5±1.6%; \( P=0.271 \)) MO (Fig. 2H-J,V). The nkx2.1a-positive domain was unchanged (Fig. 2O-T) and no increase in cleaved caspase 3 immunoreactive cells was observed (not shown).

In rescue experiments, etv5b splice MO was co-injected with etv5b RNA (see below) and the 5-HT neuron phenotype was analysed at 72 hpf (supplementary material Fig. S2B-F). The number of 5-HT neurons was rescued to control levels (101.5±1.4%; \( P=0.494 \)) compared with uninjected control siblings; \( P=8.42\times10^{-3} \) compared with MO alone), demonstrating that the phenotype observed after etv5b MO injections is caused by specific knockdown of etv5b.
Together, we conclude that Etv5b is required for the formation of a subset of hypothalamic 5-HT neurons. In both ATG and splice etv5b morphants, however, hypothalamic 5-HT-positive cells remained, suggesting that some develop in an etv5b-independent manner.

Finally, the role of Etv5a and Pea3 in hypothalamic 5-HT neuron development was investigated by MO injection. Knockdown of Pea3, but not Etv5a, resulted in a significant reduction in the number of hypothalamic 5-HT neurons (pea3 MO: 90.9±1.8% of uninjected control siblings, P=3.4×10^{-4}; etv5a MO: 94.2±2.4%, P=0.050) (supplementary material Fig. S3A). Moreover, combinations of MOs were co-injected to target two or all three ETS factors. MOs against etv5a combined with pea3 or etv5b gave results comparable to pea3 or etv5b knockdown alone, whereas combinations including MOs against both pea3 and etv5b gave a stronger phenotype than either alone (pea3+etv5a: 89.9±3.3% of uninjected control sibling, P=0.016; etv5a+etv5b: 87.8±4.8%, P=0.027; pea3+etv5a+etv5b: 70.2±4.3%, P=1.6×10^{-7}; pea3+etv5a+etv5b: 69.4±3.1%, P=1×10^{-13}) (supplementary material Fig. S3B,C). These findings suggest that Pea3, but not Etv5a, is important for the development of hypothalamic 5-HT neurons. Further, because the combined effect of pea3 and etv5b knockdown is stronger than that of either alone, it can be suggested that they act in parallel and/or in a dose-dependent manner.

**Etv5b plays a unique role in the development of hypothalamic 5-HT neurons**

In the embryonic basal forebrain, 5-HT and DA cells are neighbouring and/or intermingled (Filippi et al., 2010; Kaslin and Panula, 2001; Yamamoto and Vernier, 2011). Moreover, there are commonalities in the metabolic pathways of catecholamines and 5-HT, and both depend on ETS-domain factors (Flames and Hobert, 2011). However, counts of the number of cells immunoreactive for TH1 in etv5b ATG or splice morphants at 72 hpf revealed no significant differences (ATG MO, cluster 4/5/6: 102.7±2.4% of uninjected control siblings, P=0.430; cluster 7: 117.8±10.1%, P=0.266; splice MO, cluster 4/5/6: 100.1±1.8%, P=0.979; cluster 7: 89.9±5.9%, P=0.209) (Fig. 3A-G). Thus, 5-HT and TH1 neurons of the basal forebrain do not share a developmental requirement for etv5b.

To further analyse the selectivity of etv5b for 5-HT neuron development, the expression of oxytocin-like [oxtl], also known as isotocin neurophysin or oxytocin (oxt) – Zebrafish Information Network, arginine vasopressin-like [avpl], also known as vasotocin neurophysin or arginine vasopressin (avp) – Zebrafish Information Network and somatostatin 3 (sst3) was analysed in morphants. These markers outline neuroendocrine cell populations (Dickmeis et al., 2007; Eaton et al., 2008) juxtaposed to the 5-HT neurons. The numbers of cells expressing oxtl (ATG MO: 96.2±2.6% of uninjected control siblings, P=0.406; splice MO: 110.2±3.5% of uninjected control siblings, P=0.103), avpl (ATG MO: 104.6±2.2%, P=0.171; splice MO: 104.2±2.4%, P=0.257) or sst3 (ATG MO: 99.6±2.2%, P=0.883; splice MO: 98.4±2.3%, P=0.663) within the basal forebrain were unaffected at 72 hpf (Fig. 3H-X). These findings suggest that, among the different neuronal populations investigated, etv5b plays a unique role in the development of hypothalamic 5-HT neurons.

**Etv5b is sufficient to induce 5-HT identity within a specific competence field in the basal forebrain**

To address whether Etv5b is sufficient for induction of the hypothalamic 5-HT identity, wild-type eggs were injected with increasing concentrations of etv5b RNA and the number of 5-HT cells was assessed at 72 hpf. At all RNA concentrations tested a significant increase in cell numbers was observed (25 ng/µl: 116.4±2.3% of uninjected control siblings, P=1.1×10^{-5}; 50 ng/µl: 110.6±2.6%, P=5.39×10^{-3}; 100 ng/µl: 112.7±2.6%, P=7.8×10^{-4}; 200 ng/µl: 110.2±3.1%, P=0.012) (Fig. 4A,B,E). By contrast, in the r. population no consistent changes in numbers were found (25 ng/µl: 102.0±1.4% of uninjected control siblings, P=0.271; 50 ng/µl: 106.8±1.7%, P=1.9×10^{-3}; 100 ng/µl: 103.2±1.6%, P=0.105; 200 ng/µl: 106.3±2.3%, P=0.018) (Fig. 4C,D,E). These results demonstrate an inductive effect of etv5b on the development of hypothalamic 5-HT neurons. Interestingly, the supernumerary 5-HT-positive cells were located within the normal hypothalamic domains and were never observed at ectopic sites. This suggests that Etv5b acts on preselected cells or within a hypothalamic competence field responsive to its activity.

**The development of hypothalamic 5-HT cells depends on Fgf signalling**

Etv5b is a target of Fgf signalling in various contexts (Guillenmot and Zimmer, 2011). To confirm that this is also the case in the hypothalamus, Fgf signalling was blocked by application of SU5402, an Fgf receptor tyrosine kinase activity inhibitor (Raible and Brand, 2001), at the 3-somite stage (ss)-24 hpf, 24-36 hpf or 36-48 hpf, and etv5b expression was analysed immediately after blockade. No transcripts for etv5b were detected in the hypothalamus after SU5402 treatment (Fig. 5A,B; supplementary material Fig. S4A-D). SU5402 treatment (24-36 hpf) also abolished hypothalamic etv5a and pea3 expression (supplementary material Fig. S4E-H).

To determine if and when Fgf signalling is required for 5-HT neuron formation, we blocked Fgf signalling in wild-type embryos at different developmental intervals chosen to cover phases of proliferation, differentiation and maturation of hypothalamic 5-HT neurons by exposure to SU5402. After drug treatment, the embryos were left in control medium until 72 hpf. No morphological or positional differences in the 5-HT-positive cells were observed after SU5402 exposure (Fig. 5C,D). However, a significant reduction in the number of 5-HT-immunoreactive cells in clusters i.p. was observed for treatments until 48 hpf (Fig. 5G, Table 1). By contrast, 5-HT-positive cell numbers in the r. population were unchanged for all conditions (Fig. 5E-G, Table 1). These effects were observed in the absence of anomalies in the growth or morphogenesis of the basal forebrain, as assessed by nks2.1a expression at 48 hpf (Fig. 5H-O). The effects observed after each of these treatments are of the same order of magnitude as those resulting from etv5b knockdown. We could not determine whether there was a temporal additive effect of Fgf blockade on the number of 5-HT cells because in embryos treated with SU5402 between 3 ss and 72 hpf the overall size of the nks2.1a-positive domain was reduced (not shown). However, some 5-HT cells always remained despite long-term blockade of Fgf signalling, suggesting that, as upon abrogation of Etv5b function, a subset of the 5-HT neurons develops independently of Fgf activity.

Finally, the influence of Fgf signalling on the Hh and Notch pathways was explored by SU5402 treatment (24-48 hpf) of wild-type or Notch reporter transgenic [Tg(Tp10glob:eGFP)] (Parsons et al., 2009) embryos followed by detection of transcripts for patched 1 (tpc1) or gfp, respectively. No changes in expression levels were observed suggesting that, within the hypothalamus, Fgf signalling does not regulate Hh or Notch signalling between 24 and 48 hpf (supplementary material Fig. S5).

DEVELOPMENT
To investigate whether the effect of Fgf signalling is on 5-HT progenitors, we analysed the presence of hypothalamic Fgf receptor (*fgfr1a*, 2, 3 and 4) transcripts. All receptor genes were detectable during the time window when the 5-HT clusters are developing (supplementary material Fig. S6A-H); however, only *fgfr1a*, 2 and 4 were found in the caudal hypothalamus where 5-HT clusters i.p. are located later in development. Further, *fgf3* and *fgf8a* (supplementary material Fig. S6I-N), but not *fgf8b* (not shown), were detectable in the basal forebrain starting from 24 hpf and later in the caudal hypothalamus. These findings support a role of Fgf signalling during hypothalamic 5-HT neuron development.

To evaluate whether *etv5b* mediates Fgf signalling when controlling the development of hypothalamic 5-HT neurons, we attempted to rescue the effect of SU5402 treatment by *etv5b* overexpression. *etv5b* RNA was injected at the one-cell stage, the embryos were then treated with SU5402 between 24 and 36 hpf, and analysed at 72 hpf. As expected, the number of 5-HT-positive cells in clusters i.p. was reduced in embryos treated only with SU5402 (79.2±2.3% of DMSO-treated control siblings; *P*=7.1×10⁻⁷). *etv5b* RNA-injected embryos showed the expected increase in 5-HT cell numbers (107.6±2.3%; *P*=0.040). In embryos injected with *etv5b* and treated with SU5402, the number of 5-HT neurons (90.7±4.5% of DMSO-treated control siblings; *P*=0.085) was significantly greater than with SU5402 treatment alone (*P*=0.030), although the increase did not reach the level achieved by *etv5b* overexpression alone (*P*=2.06×10⁻³) (Fig. 6A-D,I). In all cases, 5-HT cell numbers in the r. population were unchanged (SU5402 only: 101.3±2.5% of DMSO-treated control siblings, Fig. 3).
Fig. 4. Etv5b is sufficient to induce 5-HT identity within a specific competence field in the hypothalamus. (A-D) Confocal maximum intensity projections showing a control zebrafish embryo and an embryo injected with etv5b RNA and processed for 5-HT immunohistochemistry. The intermediate/posterior (i./p.) clusters of the hypothalamus (A,B) and the 5-HT population of the anterior raphe (r.) are indicated (C,D). Ventral views, anterior left. Scale bar: 50 µm. (E) The number of 5-HT cells in the i./p. and r. clusters at 72 hpf after injection of increasing concentrations of etv5b RNA expressed as percentage of control. n, total number of embryos analysed for each experiment. *P≤0.05; n.s., not significant.

P=0.759; etv5b RNA alone: 102.2±2.3%, P=0.588; double exposed: 100.0±3.0, P=1.00) (Fig. 6E-I). Together, these results suggest that the effect of Fgf signalling activity on 5-HT cell formation in the hypothalamus is, at least in part, mediated by Etv5b. The lack of complete rescue by etv5b upon SU5402 treatment might indicate that Fgf acts via additional targets to control the development of hypothalamic 5-HT neurons.

Etv5b regulates cell cycle progression in neural progenitors of the basal forebrain

Fgf signalling and Etv5b could be required for the proliferation, differentiation, maturation and/or survival of 5-HT precursors or neurons. The proliferation status and fate of hypothalamic neural precursors were monitored in etv5b ATG morphants. At 36 hpf, a developmental stage when 5-HT progenitors are actively...

Fig. 5. Development of hypothalamic 5-HT neurons depends on Fgf signalling. (A,B) Micrograph showing DMSO control and SU5402-exposed zebrafish embryos processed for etv5b ISH. Following SU5402 treatment, no etv5b transcripts were detected in the hypothalamus (h). Lateral view, anterior left. (C-F) Confocal maximum intensity projections showing control and SU5402-exposed embryos processed for 5-HT immunohistochemistry. The intermediate/posterior (i./p.) clusters of the hypothalamus (C,D) and the 5-HT population of the anterior raphe (r.) (E,F) are indicated. Ventral views, anterior left. (G) The number of 5-HT cells after SU5402 exposure during the indicated time intervals in clusters i./p. and r. at 72 hpf expressed as percentage of control (dashed line at 100%). *P≤0.05; n.s., not significant. (H-O) nkx2.1a expression in DMSO controls and SU5402-exposed embryos. Lateral (H-K) and ventral (L-O) views, anterior left. Scale bars: 50 µm.
proliferating (supplementary material Fig. S7), the embryos were exposed to one pulse of BrdU and fixed (Fig. 7A). Cells in S or M phase were identified by their incorporation of BrdU or expression of phosphohistone H3 (ph-H3), respectively. The number of BrdU-positive cells increased in the hypothalamus in morphants, whereas the number of mitotic cells decreased (Fig. 7B,D-G, Table 2). The total number of cells, as evaluated by counting the number of DAPI-stained nuclei, was unchanged (data not shown). This suggests that, in morphants, hypothalamic progenitor cells might display a longer S phase or be blocked in S phase. Thus, normally, Etv5b might exert a positive effect on S-phase progression or influence the transition from S to G2 phase.

To further investigate the role of Etv5b in cell cycle progression, we used the Fucci transgenic lines expressing different fluorescent reporter molecules fused with the ubiquitylation domains of either human geminin (mAG-hGem), which peaks during S-G2-M, or zebrafish Cdt1 (mKO2-zCdt1), with highest expression during G1 (Sakaue-Sawano et al., 2008; Sugiyama et al., 2009). Double-transgenic eggs were injected with the etv5b ATG MO and analysed for the S-G2-M and G1 markers at 36 hpf (Fig. 7A). Morphants exhibited a 2.1-fold increase in the total number of hypothalamic proliferating (S-G2-M-G1) cells (Fig. 7C,H-K, Table 2). An analysis of the cells in the different phases additionally revealed a proportionally larger increase in cells in S-G2-M (2.7-fold) than in G1 (1.9-fold) in morphants. No significant difference was found in the total number of cells (DAPI-stained nuclei, data not shown). These results confirm the above interpretation that Etv5b positively influences cell cycle progression during S phase or during the transition from S to G2.

**Etv5b is required to maintain 5-HT progenitors in a proliferative state**

To determine how the impaired cell cycle kinetics resulting from Etv5b abrogation impacts on 5-HT differentiation, we analysed the fate of hypothalamic progenitors in etv5b ATG morphants. Embryos were treated with a pulse of BrdU at 36 hpf followed by a chase until 72 hpf, when they were processed for BrdU and 5-HT double immunohistochemistry (Fig. 8A). We observed a significantly higher proportion of 5-HT cells originating from BrdU-positive progenitors in morphants (157.8±6.2% of uninjected control siblings; $P = 4 \times 10^{-6}$) (Fig. 8B-H). This is in line with the above experiments in which etv5b abrogation results in a longer/blocked S phase at 36 hpf, and further indicates that, following this delay, BrdU-labelled progenitors at 36 hpf eventually exit the cell cycle to differentiate with a bias toward a 5-HT fate.

Because the final outcome of etv5b loss-of-function is fewer hypothalamic 5-HT neurons, we tested whether this 36-hpf phenotype could secondarily impact on later 5-HT precursors. The

**Table 1. Quantification of 5-HT-immunoreactive neurons after Fgf signalling blockade**

<table>
<thead>
<tr>
<th>Cluster</th>
<th>Stage</th>
<th>5-HT cell number (%) with SU5402</th>
<th>$P$-value</th>
<th>$n$</th>
</tr>
</thead>
<tbody>
<tr>
<td>i./p.</td>
<td>3-15 ss</td>
<td>71.6±3.9</td>
<td>$9 \times 10^{-8}$</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>15-20 ss</td>
<td>76.6±4.1</td>
<td>$2 \times 10^{-5}$</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>20 ss-24 hpf</td>
<td>80.2±2.9</td>
<td>$2 \times 10^{-5}$</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>24-36 hpf</td>
<td>61.6±2.3</td>
<td>$9 \times 10^{-13}$</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>36-48 hpf</td>
<td>91.5±2.7</td>
<td>0.040</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>48-72 hpf</td>
<td>100.8±2.8</td>
<td>0.858</td>
<td>18</td>
</tr>
<tr>
<td>r.</td>
<td>3-15 ss</td>
<td>96.2±2.1</td>
<td>0.223</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>15-20 ss</td>
<td>95.7±3.0</td>
<td>0.240</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>20 ss-24 hpf</td>
<td>96.0±2.0</td>
<td>0.214</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>24-36 hpf</td>
<td>94.2±2.3</td>
<td>0.147</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>36-48 hpf</td>
<td>98.2±1.6</td>
<td>0.535</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>48-72 hpf</td>
<td>96.7±1.8</td>
<td>0.191</td>
<td>20</td>
</tr>
</tbody>
</table>

5-HT-immunopositive cell numbers are shown as a percentage of the mean value calculated from DMSO-treated control siblings (mean ± s.e.m.). $P$-value is by Student's $t$-test.

**Fig. 6. Etv5b mediates Fgf signalling in the hypothalamus.** Rescue experiment in which zebrafish eggs were injected with etv5b RNA and then treated with SU5402 (24-36 hpf). These embryos were compared with etv5b overexpression alone or SU5402 exposure alone. Embryos exposed to DMSO served as controls. (A-H) Confocal maximum intensity projections showing embryos subjected to the indicated treatments and processed for 5-HT immunohistochemistry. The intermediate/posterior (i./p.) clusters of the hypothalamus (A-D) and the 5-HT population of the anterior raphe (r.) are indicated (E-H). Ventral views, anterior left. Scale bar: 50 μm. (I) The number of 5-HT cells in the i./p. and r. clusters at 72 hpf after the indicated treatments expressed as percentage of control. *$P$$\leq$0.05; n.s., not significant.
chase experiment described above was repeated, but with a BrdU pulse at 45 hpf (Fig. 8A). This resulted in a significantly lower proportion of BrdU-labelled cells among the remaining 5-HT cells in morphants (63.7±3.3% of uninjected control siblings; \( P = 3 \times 10^{-7} \)) (Fig. 8B,I-N). These observations suggest that the 5-HT progenitor population, following a transient cell cycle delay, is depleted in etv5b morphants after 36 hpf, resulting in an overall decrease in the number of 5-HT neurons at 72 hpf.

These findings show that Etv5b, probably through the control of cell cycle parameters, is required to maintain 5-HT progenitors of the hypothalamus and thereby provide sustainable generation of 5-HT cells.

**DISCUSSION**

Here we demonstrate that Fgf signalling, acting via the ETS-domain transcription factor Etv5b, directs the development of hypothalamic 5-HT neurons. Mechanistically, Etv5b controls the cell cycle kinetics of proliferating 5-HT progenitors by promoting their transition through S phase or from S to G2 phase, and this effect impacts on their 5-HT fate acquisition. Considering that the ETS-domain transcription factor Pet1 is a determinant of 5-HT identity in the raphe nuclei, our results highlight the prominent role of ETS-domain factors for 5-HT development within distinct brain subdivisions. These factors, however, appear to target different steps along the 5-HT commitment and differentiation cascade.

**Etv5b controls the final number of 5-HT neurons**

We show that Etv5b influences the final number of hypothalamic 5-HT neurons. Interestingly, Etv5b has a broad expression domain compared with where mature 5-HT cells are found, indicating that not only progenitors of the 5-HT population but also others express Etv5b. Next to the basal forebrain 5-HT cells, cells positive for avpl, oxtl, sst3 or TH1 are found (Dickmeis et al., 2007; Eaton et al., 2008; Filippi et al., 2010; Kaslin and Panula, 2001; Yamamoto et al., 2011). None of these markers was reduced in etv5b morphants, suggesting that etv5b has a different function in non-5-HT cells or that its function is compensated for by other factors.

**Etv5b regulates the cell cycle of 5-HT progenitors**

A major finding is that Etv5b controls cell cycle properties in 5-HT progenitors, with an impact on their mature fate. We highlight impaired cell cycle progression in etv5b morphants, when a high proportion of 5-HT precursors are still in proliferation, followed by a dramatic decrease in the proportion of later progenitors acquiring the 5-HT fate. Although we cannot exclude the possibility that Etv5b has a role later in development (e.g. during 5-HT precursor
Table 2. Analysis of cell cycle markers after etv5b knockdown

<table>
<thead>
<tr>
<th>Cell cycle phase (marker)</th>
<th>etv5b morphant cell numbers (%)</th>
<th>P-value</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>S (BrdU)</td>
<td>144.2±10.2 1.19×10^{-3}</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>M (ph-H3)</td>
<td>57.5±4.9 4×10^{-5}</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>S-G2-M-G1 (Fucci total)</td>
<td>207.0±27.6 3.04×10^{-3}</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>S-G2-M (mAG:hGem)</td>
<td>271.7±45.2 3.44×10^{-2}</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>G1 (mK02::Cdtd1)</td>
<td>189.3±25.9 6.46×10^{-2}</td>
<td>11</td>
<td></td>
</tr>
</tbody>
</table>

Cell numbers are shown as a percentage of the mean value calculated from uninjected control siblings (mean ± s.e.m.). P-value is by Student’s t-test.

differentiation or survival), these observations suggest that the primary impact on 5-HT numbers results from this early role of Etv5b. This interpretation is also in line with the fact that SU5402 treatments have no effect when applied during 5-HT differentiation. Our proliferation analyses argue for a lengthened S phase or transition from S to G2 phase in the absence of Etv5b at 36 hpf, followed by increased 5-HT differentiation. Recently, a correlation has been shown between S-phase duration and the occurrence of neurogenic versus amplifying progenitor divisions in the mouse (Arari et al., 2011). In our system, it is possible that a lengthened S phase renders more progenitors sensitive to 5-HT commitment cues operating at this stage. We propose that the pool of 5-HT progenitors remaining in cycle for subsequent events of 5-HT neuron generation is consequently depleted, with an overall decrease in the number of 5-HT neurons generated. The fact that fewer 5-HT neurons are produced from progenitors pulsed at 45 hpf is in agreement with this interpretation.

There is prior evidence that Etv5b may regulate the expression of genes that encode cell cycle control proteins (Coletta et al., 2004; Hess et al., 2006; Kurpios et al., 2003). For instance, a transcriptome microarray analysis on mouse tumour cells subjected to Etv5 (Erm) knockdown identified cyclin D1 and D2 as targets (Firlej et al., 2008). However, the roles of Etv5 in proliferation are still unclear. For example, expression of a dominant repressor Etv5 in developing mouse lung epithelium enhanced proliferation (Liu et al., 2003), whereas neural crest cells exhibit reduced proliferation upon expression of dominant negative Etv5 (Paratore et al., 2002). Our findings support the notion of a positive effect of Etv5b on proliferation in the nervous system, and suggest that transcriptional targets might also be found among cell cycle regulators that act during S phase. As proposed by others, Etv5b could also control the cell cycle characteristics of progenitor cells indirectly by regulating the expression of chemokines acting in an autocrine/paracrine manner (Chen et al., 2005).

Overall, these studies show that ETS-domain transcription factors influence proliferation and cell cycle control, although some context-dependent variations are plausible. Our findings more specifically demonstrate that the S phase is a target of Etv5b in proliferating 5-HT progenitors.

Hypothalamic and hindbrain neurons depend on ETS-domain factors during development

Pet1 is known to play a role in the maturation and maintenance of raphe 5-HT neurons (Hendricks et al., 1999; Liu et al., 2010). Interestingly, pet1 is only expressed in hindbrain 5-HT neurons in zebrafish (Lillesaar et al., 2007), and our attempts to identify a paralogous gene remain unsuccessful. In the current study we hypothesized that another ETS-domain transcription factor could play a similar role in the hypothalamus to Pet1 in the hindbrain. Our results confirm the hypothesis that an ETS-domain transcription factor controls the development of hypothalamic 5-HT neurons. Moreover, 5-HT development and pet1 expression are controlled by Fgf signalling, albeit indirectly (Kiyasova and Gaspar, 2011). However, the action of Pet1 and of Etv5b appear fundamentally different. In the hindbrain, pet1 is expressed by postmitotic cells only, and its expression is restricted to cells that are, or will become, serotonergic (Hendricks et al., 1999). Further, Pet1 directly controls the expression of 5-HT identity genes. By contrast, etv5b is expressed by proliferating 5-HT precursors before the onset of 5-HT identity genes. In addition, etv5b expression is not restricted to presumptive 5-HT cells. It therefore seems unlikely that Etv5b would directly control 5-HT identity genes, although we cannot exclude the possibility that Etv5b has additional functions later in development. Indeed, it is expressed in the adult hypothalamus (Topp et al., 2008) where 5-HT cells are located, arguing for later roles. Studies using SU5402
Fig. 9. Model for the role of Fgf signalling and Etv5b in the generation of hypothalamic 5-HT neurons during zebrafish embryonic development. The domain of the hypothalamus hosting the intermediate/posterior (i./p.) clusters of 5-HT neurons is indicated. The influence of Fgf signalling is apparent until 48 hpf. Fgf signalling controls the expression of etv5b in 5-HT progenitors, and Etv5b influences the number of mature 5-HT neurons. The function of Etv5b is restricted to a competence field and/or competent progenitors within the hypothalamus (grey circle). Additional signalling pathways, which could be other Fgf downstream targets and/or non-Fgf cues, that are necessary for acquiring the 5-HT identity are hypothesized (X). Etv5b regulates cell cycle kinetics before the 5-HT differentiation stage by having a positive effect on the progression through S phase and/or on the transition from S to G2 phase. The action of Etv5b on the cell cycle is likely to be indirect via transcriptional regulation of one or several cell cycle control molecules (Y).

Our findings indicate that not all 5-HT neurons require Fgf/Etv5b for their development. Indeed, a number of 5-HT neurons still develop despite Fgf or Etv5b abrogation. This might relate to expression of Etv5b in a subset of 5-HT precursors, as suggested using the ermp:gy tracer line. Alternatively, 5-HT precursors might differ in their sensitivity to Etv5b function. All the major signalling pathways are active in the basal forebrain of zebrafish (Machluf et al., 2011). One or several of these pathways might be of importance for the 5-HT cells remaining after Fgf and Etv5b perturbation. Overall, the partial dependency on Etv5b is reminiscent of the incomplete effect of Pet1 knockout on the raphe 5-HT population in mouse: in the absence of Pet1, a subset of the raphe neurons still expresses 5-HT identity genes, although at lower levels. Interestingly, these remaining 5-HT cells exhibit a specific axonal projection profile, suggesting that raphe nuclei in mouse host two genetically and functionally distinct 5-HT populations (Kiyasova et al., 2011). It will be interesting to see whether the distinct requirement that we observe for Etv5b in the hypothalamus also highlights functionally distinct groups of 5-HT neurons.

Finally, we demonstrate that the ubiquitous expression of Etv5b is not capable of inducing ectopic 5-HT neurons, but increases the number of neurons formed within normal hypothalamic 5-HT clusters. Mechanistically, given the effect of Etv5b on the proliferation kinetics of 5-HT precursors, it is possible that increased Etv5b expression acts on these same precursors to accelerate their proliferation, leading to an increased number of 5-HT progeny. The spatially limited competence of Etv5b could be explained by the requirement for Fgf-induced phosphorylation of Etv5b for its activation and subsequent binding to DNA for transcriptional control of target genes (Sharrocks, 2001). Thus, Etv5b is only active in places where Fgf signalling is present. However, the lack of ectopic cells in the etv5b overexpression situation, including areas with active Fgf signalling such as the telencephalon or midbrain-hindbrain boundary region, suggests that additional factors and signalling pathways are required to generate 5-HT cells.

In conclusion, our results permit, for the first time, a model to be proposed for the generation of hypothalamic 5-HT cells in a vertebrate (Fig. 9). Our experiments show that Fgf signalling regulates the number of 5-HT cells via Etv5b by acting before the 5-HT differentiation stage. Supporting a role for Fgf signalling, we found expression of fgb3 (and fga8a) in the caudal hypothalamus. Furthermore, preliminary findings in the fgb3 mutant [lia (Herzog et al., 2004)] revealed a loss of 5-HT neurons in the i./p. clusters at 72 hpf (not shown). Owing to severe and widespread developmental brain defects, a similar analysis could not be performed on the fga8a mutant [ace (Reifers et al., 1998)]. These findings make Fgb3 an interesting candidate for future investigations.

Further, overexpression experiments highlight the importance of competence to respond to Etv5b function. They might indicate the existence of preselected progenitors and/or a competence field. Our analysis of cell cycle markers in the etv5b loss-of-function
experiment shows that Etv5b positively influences cell cycle progression within the 5-HT progenitor population, an effect that is required to maintain the pool of 5-HT progenitors necessary for the generation of a normal number of 5-HT cells. This provides an interesting mechanism for how a gene that is expressed broadly in development can modify the number of mature cells in a restricted population during a critical time point. This might be clinically relevant considering the many psychiatric disorders that are suggested to originate from subtle developmental changes affecting the 5-HT circuits (Gross and Hen, 2004; Scott and Deneris, 2005).

Acknowledgements
We thank members of the laboratories of L.B.-C., P.G. and Dr Philippe Vernier for support and input. Fixed 4a embryos were kindly provided by Sandra Leibold.

Funding
This work was supported by Agence Nationale de la Recherche [ANR6065-neur-046], Fyson Foundation [C.I.L.], a post-doctoral fellowship from the Foundation for Medical Research [SPF20110421420 to A.B.]; and the Schlemberger Association [DLS/GP/090305 to L.B.-C.]. C.I.L. was supported by Chair of Excellence [ANR-08-CEXC-001-01]. The teams of L.B.-C. and P.G. are part of the Ecole de Neurosciences de Paris Il de France (ENP).

Competing interests statement
The authors declare no competing financial interests.

Supplementary material
Supplementary material available online at http://dev.biologists.orglookup/suppl?doi=10.1242/dev.089094/-/DC1

References


