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Fgf15-mediated control of neurogenic and proneural gene expression regulates dorsal midbrain neurogenesis

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ABSTRACT

The balanced proliferation and cell cycle exit of neural progenitors, by generating the appropriate amount of postmitotic progeny at the correct time and in the proper location, is required for the establishment of the highly ordered structure of the adult brain. Little is known about the extrinsic signals regulating these processes, particularly in the midbrain. Fibroblast growth factor (Fgf) 15, the mouse ortholog of FGF19 and member of an atypical Fgf subfamily, is prominently expressed in the dorsolateral midbrain of the midgestational mouse embryo. In the absence of *Fgf15*, dorsal midbrain neural progenitors fail to exit the cell cycle and to generate the proper amount of postmitotic neurons. We show here that this is due to the altered expression of inhibitory/neurogenic and proneural/neuronal differentiation helix-loop-helix transcription factor (TF) genes. The expression of *Id1*, *Id3*, and *Hes5* was strongly increased and ectopically expanded, whereas the expression of *Ascl1* (*Mash1*), *Neurog1* (*Ngn1*) and *Neurog2* (*Ngn2*) was strongly decreased and transcription of *Neurod1* (*NeuroD*) was completely abolished in the dorsolateral midbrain of $Fgf15^{-/-}$ mice. These abnormalities were not caused by the mis-expression of cell cycle regulatory proteins such as cyclin-dependent kinase inhibitors or retinoblastoma proteins. Furthermore, human FGF19 promotes cell cycle exit of murine dorsal neural progenitors *in vitro*. Therefore, our data suggest that Fgf15 is a crucial signaling molecule regulating the postmitotic transition of dorsal neural progenitors and thus the initiation and proper progression of dorsal midbrain neurogenesis in the mouse, by controlling the expression of neurogenic and proneural TFs.

Introduction

The establishment of the complex and highly ordered structure of the adult mammalian brain also requires a precisely orchestrated interplay of extrinsic and intrinsic signals during development, that regulate the spatial and temporal balance between self-renewal and cell cycle exit of neural progenitors and the generation of the appropriate numbers of postmitotic progeny. Among the intrinsic signals are TFs of the helix–loop–helix (HLH) family, playing a particularly prominent role in the control of progenitor proliferation, cell cycle exit and neuronal differentiation (Guillemot, 2007). These TFs are therefore classified in three groups: Proneural TFs (Ascl1 (Mash1) and Neurogenins (Neurog, Ngn)) promote the cell cycle exit of neural progenitors and initiation of neurogenesis, and the activation of Notch signaling in adjacent progenitors. Neuronal differentiation TFs (Neurod1 (NeuroD)) are induced by the proneural TFs in postmitotic cells and control the neuronal differentiation program. Inhibitory or neurogenic TFs (Id and Hes) directly inhibit proneural TFs or repress proneural gene expression, thereby maintaining the proliferative, undifferentiated state of neural progenitors. Cell cycle proteins, such as the cyclin-dependent kinase inhibitor (cdki) Cdkn1b ($p27^{Kip1}$) and the retinoblastoma (Rb) proteins Rb1, Rbl1 (p107) and Rbl2 (p130), are another type of intrinsic factors promoting cell cycle exit and differentiation of neural progenitors (Galderisi et al., 2003; Nguyen et al., 2006).

In contrast to the intrinsic factors, the extrinsic signals acting upstream of these TFs or cell cycle proteins are not yet fully established, particularly in the murine midbrain. These are mostly secreted factors, such as bone morphogenetic protein (BMP)/transforming growth factor β (Tgfβ) family members promoting cell cycle exit of neural progenitors (Roussa

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et al., 2006; Siegenthaler and Miller, 2005); Wnt family members maintaining the proliferative state of neural progenitors, repressing their differentiation, or promoting neurogenesis and neuronal fate specification in a time- and context-dependent manner (Hirabayashi et al., 2004; Kuwabara et al., 2009; Megason and McMahon, 2002; Wexler et al., 2009); and members of the fibroblast growth factor (Fgf) family, which so far have been mostly implicated in the maintenance of the proliferative neural progenitor state (Mason, 2007).

The Fgf family comprises 22 members in mammals, classified in seven subfamilies (Itoh and Ornitz, 2008; Mason, 2007). Fgf15/19, Fgf21 and Fgf23 constitute an atypical Fgf subfamily based on their evolutionary relationship and on the facts that these Fgfs are transcriptionally regulated by members of the nuclear receptor class of ligand activated TFs, possess low-affinity heparin-binding sites and therefore act in an endocrine manner, and require Klotho/β-Klotho transmembrane proteins for efficient signaling via Fgf receptors (Itoh and Ornitz, 2008; Jones, 2008). Mouse Fgf15, despite its low amino acid identity (between 32% and 51%), is considered the structural and functional ortholog of human, chick and zebrafish FGF19/Fgf19 (Miyake et al., 2005; Nishimura et al., 1999; Wright et al., 2004). *Fgf15* expression is confined mostly to the developing central nervous system (CNS) (Gimeno et al., 2003; Ishibashi and McMahon, 2002; McWhirter et al., 1997), but its function was first described in non-neural tissues, where it controls bile acid homeostasis, gall bladder filling and proper morphogenesis of the cardiac outflow tract (Choi et al., 2006; Inagaki et al., 2005; Vincentz et al., 2005). However, a recent report indicated that Fgf15 suppresses proliferation and promotes neural differentiation during neocortical development, although the mechanism of this proneural activity of Fgf15 remained unclear (Borello et al., 2008). Here we show that extrinsic Fgf15 is a crucial signaling molecule regulating the expression of intrinsic inhibitory/neurogenic and proneural HLH TFs in the mouse midbrain, thereby controlling the cell cycle exit of dorsal neural progenitors and their differentiation into neurons.

Materials and methods

Mouse strains

CD-1 mice were purchased from Charles River (Kisslegg/Germany). Generation and genotyping of *Fgf15*$^{+/-}$ mice in a mixed C57BL/6-129/Sv background was described by Wright et al. (2004). These mice were outcrossed with CD-1 mice for 12 generations (F12). *Fgf15*$^{-/-}$ embryos were analyzed from F1 onwards and phenotypic differences were not detected between F1 and F12 embryos. Collection of embryonic stages was done from timed-pregnant females of heterozygous (*Fgf15*$^{+/-}$) intercrosses, noon of the day of vaginal plug detection was designated as embryonic day 0.5 (E0.5). Embryos were additionally staged according to Theiler (1989). Mutant embryos were always compared to their wild-type (*Fgf15*$^{+/+}$) littermates and at least 3 embryos were analyzed for each probe, genotype and stage, if not otherwise indicated in the text. Animal treatment was conducted under federal guidelines and was approved by the HMGU Institutional Animal Care and Use Committee.

Radioactive in situ hybridization (ISH)

Paraffin sections (8 μm) were processed for radioactive ([α-^{35}S]UTP, Amersham/UK) ISH as described in Fischer et al. (2007). Riboprobes used were *Fgf15* (McWhirter et al., 1997), *Fgf8*, *Shh*, *Pax6* and *En1* (Puelles et al., 2003), *Wnt1* (Fischer et al., 2007), *Wnt3a* (Parr et al., 1993), *Id1* (Benezra et al., 1990), *Id3* (Christy et al., 1991), *Hes5* (Akazawa et al., 1992), *Hes3* (Hirata et al., 2001), *Ascl1* (*Mash1*), *Neurog1* (*Ngn1*), *Neurog2* (*Ngn2*) and *Neurod1* (*NeuroD*) (Cau et al., 1997; Ma et al., 1997), Helt (Mgn) (Guimera et al., 2006a), *Dll1* (Bettenhausen et al., 1995), *Dusp6* (*Mkp3*) (Echevarria et al., 2005), *Fgfr1-3* (Blak et al., 2005), *Spry2* (Minowada et al., 1999), *Etv4* (*Pea3*) and *Etv5* (*Erm*) (Blak et al., 2007). Images were taken with an Axioplan2 microscope or StemiSV6 stereomicroscope using bright- and dark-field optics, AxioCam MRc camera and Axiovision 4.6 software (Zeiss/Germany), and processed with Adobe Photoshop 7.0 or CS software (Adobe Systems Inc./USA).

Immunohistochemistry (IHC)

Antigens were detected on paraffin (8 μm) or cryosections (16 μm) as reported by Brodski et al. (2003) and Puelles et al. (2004). Primary monoclonal antibodies used were mouse anti- 5-bromo-2′-deoxyuridine (BrdU) (1:10; Roche Diagnostics/Germany) and βIII-Tubulin (Tubb3, TuJ1) (1:5000; Chemicon/USA), and rat anti-Nestin (1:3; BD Pharmingen/USA). Polyclonal antisera used were rabbit anti-cleaved Caspase 3 (cCasp3) (1:200; Cell Signaling/USA), phosphorylated Histone H3 (pH3) (1:1000; Upstate/USA), Ki-67 (1:100; Vision BioSystems/UK) and Doublecortin (Dcx) (1:80, gift from O. Reiner, Weizmann Institute of Science/Israel). Secondary antibodies were either fluorescently labelled (Cy3/Cy2) or coupled to biotin/streptavidin-horseradish-peroxidase (Jackson ImmunoResearch Laboratories/USA), and detected using the VECTOR® M.O.M.™ and Vectastain ABC System (Vector Laboratories/USA). Fluorescent images were taken with an Axiovert 200 M inverted microscope (Zeiss) and processed with Adobe Photoshop 7.0 or CS software.

BrdU treatments

Intraperitoneal (i.p.) injections of pregnant dams with 31 μg BrdU (Sigma/Germany)/g body weight were performed on E11.5 for single (10 min) or cumulative (3× every 2 hours (hrs)) labeling. Embryos were dissected 10 min (single labeling) or 2 hrs (cumulative labeling) after the last injection and processed for immunodetection of BrdU.

Cell countings

Ki67$^+$ and pH3$^+$ cells were counted on serial coronal sections from E11.5 embryos using the Neurolucida 6 software (MBF Bioscience/USA). Cell numbers were averaged for each genotype and subjected to tests for the estimation of statistical significance as described in Statistical analyses.

Cell cycle exit assay

Pregnant dams from *Fgf15*$^{+/-}$ intercrosses were injected i.p. with BrdU (31 μg/g body weight) on E10.5 (t_0). Embryos were dissected 24 hrs later (t_{24}) at E11.5 and processed for fluorescent immunodetection of BrdU and Ki-67. Stained sections were evaluated with a confocal laser scanning microscope (LSM 510 META, Zeiss). A *z*-stack image series in intervals of 1 μm was recorded from each section comprising the entire thickness of the tissue. The index T_c of cell cycle re-entry after 24 hrs was calculated by dividing the number of Ki-67$^+$/BrdU$^+$ double-labeled cells by the total number of BrdU$^+$ cells.

Immunoblotting

The anterior neural tube (fore-/midbrain and rhombomere 1) of E11.5 embryos from *Fgf15*$^{+/-}$ intercrosses was dissected free of non-neural tissues and eye cups in ice-cold tissue lysis buffer (20 mM Tris pH7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, protease and phosphatase inhibitors (complete Mini and PhosSTOP, Roche)) and homogenized in 100 μl tissue lysis buffer. The remaining embryonic tissue was used for PCR genotyping. Total protein content was determined by Bradford assay (Sigma-Aldrich/Germany). Equal amounts of total protein from tissue lysates were separated in 4–12% Criterion XT Bis-Tris Precast gels (Bio-Rad/Germany) together with controls and a biotinylated protein ladder following the manufacturer's instructions (PhosphoPlus Rb antibody kit, Cell Signaling), and blotted onto nitrocellulose membranes (Hybond-ECL, GE Healthcare Europe/