FGF SIGNALING IN NEUROGENESIS AND PATTERNING OF THE DEVELOPING MIDBRAIN AND ANTERIOR HINDBRAIN

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SUMMARY

Embryonic midbrain and hindbrain are structures which will give rise to brain stem, pons and medulla in the adult vertebrates. These brain regions contain several nuclei which are essential for the regulation of movements and behavior. They include serotonin-producing neurons, which develop in the hindbrain, and dopamine-producing neurons in the ventral midbrain. Degeneration and malfunction of these neurons leads to various neurological disorders, including schizophrenia, depression, Alzheimer's, and Parkinson's disease. Thus, understanding their development is of high interest.

During embryogenesis, a local signaling center called isthmic organizer regulates the development of midbrain and anterior hindbrain. It secretes peptides belonging to fibroblast growth factor (FGF) and Wingless/Int (Wnt) families. These factors bind to their receptors in the surrounding tissues, and activate various downstream signaling pathways which lead to alterations in gene expression. This in turn affects the various developmental processes in this region, such as proliferation, survival, patterning, and neuronal differentiation.

In this study we have analyzed the role of FGFs in the development of midbrain and anterior hindbrain, by using mouse as a model organism. We show that FGF receptors cooperate to receive isthmic signals, and cell-autonomously promote cell survival, proliferation, and maintenance of neuronal progenitors. FGF signaling is required for the maintenance of *Sox3* and *Hes1* expression in progenitors, and Hes1 in turn suppresses the activity of proneural genes. Loss of *Hes1* is correlated with increased cell cycle exit and premature neuronal differentiation. We further demonstrate that FGF8 protein forms an antero-posterior gradient in the basal lamina, and might enter the neuronal progenitors via their basal processes.

We also analyze the impact of FGF signaling on the various neuronal nuclei in midbrain and hindbrain. Rostral serotonergic neurons appear to require high levels of FGF signaling in order to develop. In the absence of FGF signaling, these neurons are absent. We also show that embryonic meso-diencephalic dopaminergic domain consists of two populations in the anterior-posterior direction, and that these populations display different molecular profiles. The anterior – diencephalic – domain appears less dependent on isthmic FGFs, and lack several genes typical of midbrain dopaminergic neurons, such as *Pitx3* and *DAT*. In *Fgfr* compound mutants, midbrain dopaminergic neurons begin to develop but soon adopt characteristics which highly resemble those of diencephalic dopaminergic precursors. Our results indicate that FGF signaling regulates patterning of these two domains cell-autonomously.

ABBREVIATIONS

5-HT	5-hydroxytryptophan (serotonin)
ADAM	a disintegrin and metallopeptidase
AD/HD	attention deficit hyperactivity disorder
Aldh	aldehyde dehydrogenase
Ant	adenine nucleotide translocator
A-P	antero-posterior
APC	adenomatosis polyposis coli
Aspm	asp (abnormal spindle-like), microcephaly-associated *(Drosophila)*
AVE	anterior visceral endoderm
BDNF	brain derived neurotrophic factor
bHLH	basic helix-loop-helix
BLBP	brain lipid binding protein (also called Flbp)
BMP	bone morphogenetic protein
bp	base pair(s)
Boc	biregional cell adhesion molecule-related/downregulated by oncogenes (Cdon) binding protein
BrdU	5-bromo-2´-deoxyuridine
CaMK	Calcium/calmodulin-dependent protein kinase
Cas3	caspase 3
cDNA	complimentary DNA
Cdc42	cell division cycle 42 homolog *(S. cerevisiae)*
CDK	cyclin dependent kinase
Cdo	cysteine dioxygenase
Cend	cell cycle exit and neuronal differentiation
Cep	centrosomal protein
Cgrp	calcitonin gene related polypeptide
CKI	cyclin dependent kinase inhibitor
cko	conditional knock-out; mutant
CNS	central nervous system
Comt	cathecol-O-methyltransferase
CRABP	cellular retinoic acid binding protein
Cre	Cre recombinase
Cthrc	collagen triple helix repeat containing
Dach1	dachshund 1 *(Drosophila)*
DAG	diacylglycerol
DAT	dopamine transporter
DAPI	4'-6'-diamino-2-phenylindole
Ddc	dopa decarboxylase
Dkk	dickkopf homolog *(Drosophila)*
Dll	Delta-like *(Drosophila)*
DNA	deoxyribose nuclei acid
Dusp6	dual specificity phosphatase 6 (also called Mkp3)
D-V	dorso-ventral
E	embryonic day
Ear2	eosinophil-associated, ribonuclease A family, member 2
Ednrb	endothelin receptor type B

EdU	5-ethynyl-2'-deoxyuridine
Emx	empty spiracles homolog *(Drosophila)*
En	Engrailed
ERK	extracellular-signal-regulated kinase (also called MAPK)
Erni	early response to neural induction
ES cell	embryonic stem cell
EST	expressed sequence taq
FACS	fluorescence activated cell sorting
FGF	fibroblast growth factor
Fbfpb	fibroblast growth factor binding protein
FGFR	fibroblast growth factor receptor
FRS2	fibroblast growth factor receptor substrate 2
Fst	follistatin
Fzd	Frizzled
Flrt3	fibronectin leucine rich transmembrane protein 3
Fox	forkhead box
GABA	gamma aminobutyric acid
Gas	growth arrest specific
Gata	GATA binding protein
Gbx	gastrulation brain homeobox
GDNF	glial cell line derived neurotrophic factor
GFAP	glial fibrillary acidic protein
GFP	green fluorescent protein
GLAST	glutamate aspartate transporter
Gli	GLI-Kruppel family member
Grb	growth factor receptor bound protein
Grp	gastrin releasing peptide
Hes	Hairy/Enhancer of Split *(Drosophila)*
Hesr	Hairy/Enhancer of Split related with YRPW motif (also called Hey)
Hh	Hedgehog, a family of signaling molecules
Hhip	Hedgehog interacting protein
Hook	hook homolog *(Drosophila)*
HSPG	heparan sulphate proteoglycan
HuC/D	human neuronal protein HuC/HuD
Ig	immunoglobulin
IGF	insulin-like growth factor
Igfbp	insulin-like growth factor binding protein
IHC	immunohistochemistry
Insc	inscuteable homolog *(Drosophila)*
IP3	inositol 1,4,5-trisphosphate
Irx	Iroquois related homeobox *(Drosophila)*
ISH	*in situ* hybridization
IZ	intermediate zone
JNK	c-Jun N-terminal kinase
Kcnj	potassium inwardly-rectifying channel, subfamily J
Kif	Kinesin family member
Lhx	LIM homeobox protein
Lmx1	LIM homeobox transcription factor 1
Lef	lymphoid enhancer binding factor
Lis	lissencephaly

LRP	low density lipoprotein receptor-related protein
MAb2l1	Mab-21-like-1 *(C.elegans)*
MAPK	mitogen activated protein kinase (also called ERK)
Mash	Achaete-scute complex homolog (also called Ascl) *(Drosophila)*
Math	Atonal homolog (also called Atoh) *(Drosophila)*
MEK	mitogen-activated extracellular-signal-regulated kinase kinase
Mib	Mindbomb homolog *(Drosophila)*
mINSC	inscuteable homolog *(Drosophila)*
Msx	homeobox, msh-like
MZ	mantle zone
mRNA	messenger RNA
Neurl	Neuralized homolog *(Drosophila)*
Nkx	NK transcription factor related *(Drosophila)*
Ngn	Neurogenin
NICD	Notch intracellular domain
Numa	nuclear mitotic apparatus protein
Nurr1	nuclear receptor related protein 1 (also called Nr4a4)
Olig	oligodendrocyte transcription factor
OSVZ	outer subventricular zone
Otx	orthodenticle homolog *(Drosophila)*
p75NTR	p75 neurotrophin receptor (also called Ngfr)
Pacap	pituitary adenylate cyclase-activating polypeptide (also called Adcyap1)
Pax	paired box gene
PCR	polymerase chain reaction
PDK	pyruvate dehydroxygenase kinase
Pet1	plasmacytoma expressed transcript 1
Pft	pancreas transcription factor
PH3	phosphohistone 3
Phox2	paired-like homeobox 2
PIP2	phosphatidylinositol-2-phosphate
PIP3	phosphatidylinositol-3-phosphate
PIP4	phosphatidylinositol-4-phosphate
PI3-K	phosphatidylinositol 3’ kinase
Pitx	paired-like homeobox transcription factor
PKB	protein kinase B
PKC	protein kinase C
PLC-g	phospholipase C gamma
Pou4f1	POU-domain, class 4, transcription factor 1
Prodh	proline dehydrogenase
Ptc	Patched
R26R	Rosa 26 reporter
RAR	retinoic acid receptor
RARE	retinoic acid response element
Ras	rat sarcoma
Rb	retinoblastoma
Rbpj	recombination signal binding protein for immunoglobulin kappa J region
Ret	ret proto-oncogene
Rgma	RGM domain family, member A
RGM	repulsive guidance molecule
RXR	retinoic X receptor

Sef	similar expression to Fgfs
Sfrp	secreted Frizzled-related protein
SH2	Src homology 2
Shh	Sonic hedgehog
Sim	single-minded homolog *(Drosophila)*
Sip	survival of motor neuron protein interacting protein
Six	sine oculis –related homeobox *(Drosophila)*
SMAD	MAD homolog *(Drosophila)*
Smo	Smoothened
Smurf	SMAD specific E3 ubiquitin protein ligase
SNpc	substantia nigra pars compacta
SNpr	substantia nigra pars reticulata
Sos	son of sevenless homolog *(Drosophila)*
Sost	sclerostin
Sox	SRY-box containing gene
Spry	Sprouty
SVZ	subventricular zone
TACC	transforming acidic coiled coil
Tbr	T-box brain gene
TCF	T-cell factor family transcription factor
TGF	transforming growth factor
TH	tyrosine hydroxylase
Tis21	tumor promoter inducible gene 21 (also called Btg2)
Tnfrsf	tumor necrosis factor receptor subfamily
Tob1	transducer of ErbB-2.1
Tpx2	TPX2, microtubule-associated protein homolog *(X. laevis)*
Trh	thyrotropin releasing hormone
Tuj1	neuron specific beta III tubulin
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labelling
Uncx	UNC homeobox
Vglut	vesicular glutamate transporter
Vmat	vesicular monoamine transporter
VTA	ventral tegmental area
Vtn	vitronectin
VZ	ventricular zone
WAP	whey acidic protein
Wfdc1	WAP four-disulfide core domain 1 (also called ps20)
Wif	Wnt inhibitory factor
Wnt	Wingless (Int) family
WT	wild-type
ZLI	zona limitans intrathalamica

1. REVIEW OF THE LITERATURE

1.1. Signaling pathways in the developing central nervous system

Human brain is estimated to consist of over 100 billion neurons and glial cells of different types, which form a complex network (Herrup and Williams, 1988). These cells, like all cells in the vertebrate central nervous system (CNS), have their origins in the multipotent neuroepithelial cells, which are guided towards adopting different developmental fates via intercellular signaling systems. These signals can be mediated either via cell-cell contacts, like Notch-Delta signaling, or via soluble morphogens. Morphogens in the developing nervous system belong to families of fibroblast growth factors (FGFs); Hedgehogs (Hhs); Wingless/Ints (Wnts); retinoic acid, and transforming growth factor betas (TGF-betas) (Jessell, 2000; Cayuso and Martí, 2005). They are secreted molecules, which form a gradient originating from the secreting tissue, entitled organizer or a signaling center.

According to its position in dorso-ventral (D-V) and antero-posterior (A-P) axes of the embryo, each cell receives a certain amount of each morphogen. On the cell surface, morphogens bind to their specific receptors which activate intracellular signaling pathways, leading to changes in protein activity and gene transcription. Usually the signals switch on a set of transcription factors, which act as a combination to suppress alternate neuronal fates, and promote acquisition of the correct neuronal identity. The mutual repression of the transcription factors further finetunes their expression boundaries, sharpening the domains from where differentially fated neurons arise (Jessell, 2000).

Cell's response to these signals depends on its ability of interpret them, i.e competence; and concentration and combination of the morphogens themselves. Thus the cell's position in relation to the signaling source, and earlier inductive events, affect the morphogen read-out. Furthermore, the duration of exposure to morphogens likely plays an important role, as the cells closest to the source are exposed to the signal for the longest time (Rogers and Schier, 2011). Studies on Sonic hedgehog (Shh) pathway have suggested that cells may integrate both duration and extracellular concentration of the morphogen to produce distinct intracellular responses (Ribes and Briscoe, 2009). In this case, cells dynamically refine their response to the signal, and the signaling pathway itself. Also the duration of isthmic FGF8 signaling is essential for the patterning of midbrain and anterior hindbrain structures (Sato and Joyner, 2009).

Furthermore, the signaling pathways and their downstream targets do not operate in isolation, without contact with other factors. The classic textbook models describe signaling pathways as linear routes, where ligand binding leads to receptor activation, which then activates second downstream messengers, finally leading to changes in transcription. However, the truth is more complex. In each cell, numerous signaling pathways are active simultaneously. Each pathway consists of several ligands, which display different binding affinities to receptors. These affinities are context-dependent, can be modified on several levels, and can vary greatly between cells and tissues, and between developmental stages. Furthermore, the downstream effectors interact with each other, and with components of other signaling pathways. Together with various positive and negative modulators, all these components form complex signaling

networks. The sum of all these interactions determines the net effect of signaling in each cell (Kestler et al., 2008).

For simplicity, these signaling pathways and their components are introduced here only briefly and following the traditional linear signaling models. Their function in the different stages of CNS development will be discussed in more detail in the following chapters.

1.1.1. FGF signaling

1.1.1.1. FGF ligands and receptors

FGFs were initially identified as mitogens which increased proliferation of fibroblasts *in vitro*. In mammals, 22 FGFs divided into six subfamilies have been identified (Dorey and Amaya, 2010; Itoh and Ornitz, 2011). They share a conserved core, which consists of 140 a mino acids, and show affinity to heparan sulphate proteoglycans (HSPGs). According to their mode of action, FGFs can be classified into three groups: intracellular (FGF11-14 subfamily), hormonal (FGF15/19/21/23 subfamily) and canonical (all the other four subfamilies) (Itoh and Ornitz, 2011).

Intracellular FGFs are not secreted and their function is not well understood (Goldfarb, 2005). They are known to regulate neuronal excitability via interacting with voltage-gated sodium channels (Goldfarb et al., 2007).

Hormonal FGFs function both during embryogenesis and in adults (Itoh and Ornitz, 2011). These FGFs have a very low affinity to classic FGF signaling cofactors, HSPGs, and instead bind to FGF receptors with alpha- and betaKlotho proteins, and possibly with other, yet unidentified, cofactors.

Canonical FGFs are typically secreted proteins, although FGF1 and FGF2 appear to use a Golgi-independent release mechanism (Itoh and Ornitz, 2011; Nickel, 2011). They activate signaling pathways via binding to cell surface FGF receptors together with heparan sulphate cofactors, although FGF1-3 can also be translocated into nucleus.

In the vertebrate genome, four FGF receptor genes are found, *Fgfr1-4*. They encode transmembrane tyrosine kinases, which consist of an extracellular part, containing three immunoglobulin-like (Ig-l) domains and an acid stretch between domain I and II; a transmembrane domain; and an intracellular kinase domain (**Figure 1**). The third Ig-like domain is responsible for the specificity of ligand-binding. From this domain in *Fgfr1-3*, alternative splicing generates two isoforms, IIIb and IIIc, which differ in their ligand binding affinity (Itoh and Ornitz, 2011). Thus, multiple different FGF receptors can be generated from four genes. *Fgfr1* and *Fgfr2* are both widely expressed in the developing embryo, and the corresponding mouse mutants die during early embryogenesis (reviewed in Dorey and Amaya, 2010). $Fgfr3^{null}$ mice are viable, but show skeletal defects due to excessive bone growth, and deafness due to inner ear abnormalities (Colvin et al., 2003).

In addition, *Fgfr-like1* (*Fgfrl1*, also called *Fgfr5*) gene has been identified (Trueb, 2011). In contrast to "classic" FGF receptors, FGFRL1 lacks the intracellular catalytic domain. Thus, it may function as a decoy receptor in ligand binding, this way modulating ligand presentation to the catalytic FGFRs. $Fgfrl1^{-/-}$ die perinatally due to diaphgram malfunction, and display a failure in kidney development (Trueb, 2011).

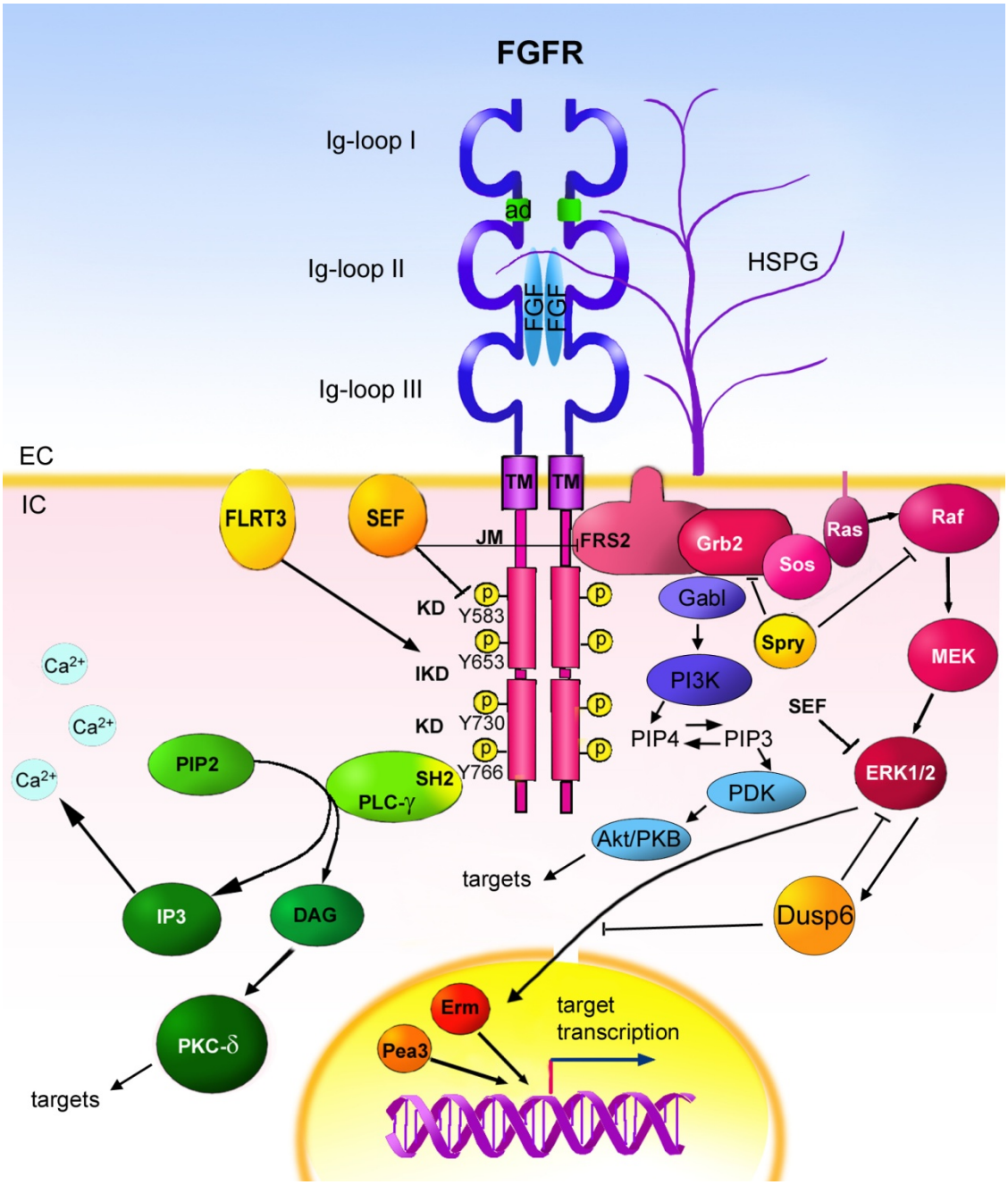


Figure 1. FGF signaling pathway.

Schematic view of FGFR-FGF-HSPG ligand complex and three major downstream signaling routes mediated by MAP-kinase (red), PI3-kinase (blue) and PLC-gamma (green). Modulators of signaling pathway, such as Sproutys and Dusp6 are shown in yellow. Canonical FGFs bind to FGFRs together with HSPGs, which induces receptor dimerization. Receptor subunits transphosphorylate each other, which activates signaling pathways and culminates in the activation of targets. Main FGFR-mediated pathway in the developing embryos is MAPK (ERK) route. Phosphorylated ERK1/2 activate nuclear ETS transcription factors such as Erm and Pea3, which regulate transcription. For simplicity, phosphorylated residues are only shown on the receptor complex. EC, extracellular space; IC, intracellular space; ad, acidic domain; TM, transmembrane domain; KD, kinase domain; JM; juxtamembrane domain; IKD, interkinase domain. Based on Thisse and Thisse (2005), Mason (2007), and Partanen (2007).

In a combination with heparan sulphate, FGF ligands bind as dimers to the extracellular domain of FGFR (Mohammadi et al., 2005). The ligand-binding induces dimerization of receptor monomers, bringing together two intracellular kinase units. These units transphosphorylate each other, which activates downstream signaling pathways (**Figure 1**). The downstream signaling events have traditionally been classified into three main pathways, which are mediated by mitogen-activated protein kinase (MAPK), PI3 kinase (PI3-K), and phospholipase C-gamma (PLC-g) (Partanen, 2007). Fibroblast growth factor receptor substrate 2 (FRS2) is an adaptor molecule which links FGF receptor activation to both MAPK and P13-K signaling pathways (Hadari et al., 2001). The relative contributions of each of these pathways vary between cell types, tissues, and developmental stages. For example, MAPK pathway has been associated with proliferation and cell fate determination, whereas PLC-g has been shown to regulate morphology and cell migration, and PI3-K to mediate cell survival (Dorey and Amaya, 2010; Partanen, 2007). Activation of MAP kinases ERK1/2 appears is a shared feature among all FGF-receptors (Mason, 2007). Although MAP kinases widely function downstream of various other signaling pathways, FGF-signaling appears to be mainly responsible for their activation in early vertebrate embryos (Christen and Slack; 1999; Tsang and Dawid, 2004; Corson et al., 2003).

1.1.1.2. Modulators of FGF signaling

The modulators of FGFR-mediated signaling are also FGF targets and form a synexpression group of genes, which contains both inhibitors and activators. The members of this group display similar expression patterns during development.

Feedback inhibitors of FGF signaling include Sef (Similar expression to *Fgfs*) family of receptor tyrosine kinase inhibitors, Sproutys, and Dual-specificity MAP kinase phosphatases (Dusps, also called Mkps). In mouse, Sefs inhibit FGFR signaling by blocking phosphorylation of the receptor and the immediate FGFR substrate (FRS2/SNT) (Kovalenko et al., 2003). Thus, Sef is able to simultaneously attenuate several pathways downstream of FGFR.

Sproutys (*Spry*), originally identified in *Drosophila*, are a highly conserved group of negative feedback regulators of FGF signaling (Mason et al., 2006). In the mammalian genome, four *Sprouty* homologs (*Spry1-4*) exist, and they function by specifically inhibiting the MAPK pathway of receptor tyrosine kinases in a cell type and growth factor -specific manner.

Dusps are able to dephosphorylate MAPK isoforms, thus rendering them inactive (Bermudez et al., 2010). Dusps involved in embryogenesis include Dusp6, 7, and 9. Of these, Dusp6 (Mkp3) shows specific ability to inactivate MAP kinases ERK1/2 (Arkell et al., 2008). It has been suggested that Dusp6 might trap the inactivated ERK1/2 in the cytosol, or transport them out of the nucleus (Bermudez et al., 2010). Dusp6 itself is a target of ERK1/2, and its phosphorylation leads to its degradation by the proteasome.

Positive modulators include Fibronectin leucine rich transmembrane proteins (Flrts) and Canopy1. Flrt proteins participate both in homotypic cell adhesion, and in the potentiation of FGF signaling by stimulating the MAPK route (Haines et al., 2006; Wheldon et al, 2010; Wei et al., 2011; Karaulanov et al., 2006). Canopy1 was recently identified as a positive regulator of FGF signaling in zebrafish (Hirate and Okamoto, 2006). It is required for both midbrain-hindbrain development and establishment of the left-right bodyplan (Hirate and Okamoto, 2006; Matsui et al., 2011).

1.1.1.3. HSPGs

HSPGs are highly sulphated glycosaminoglycans, found on the cell surface as a component of the extracellular matrix. They interact with other components of the matrix, cell adhesion molecules, and growth factors, thus affecting numerous processes, such as cell proliferation and axonal guidance, during both embryogenesis and in the adult organism (Yamaguchi, 2001). Extensive *in vitro* and *in vivo* evidence has shown the importance of HSPGs in the growth factor binding (Bernfield, 1999; Lopes et al., 2006). HSPGs affect both positively and negatively the distribution and receptor-binding of several secreted morphogens, such as Shh, FGFs, bone morphogenetic proteins (BMPs) and Wnts (Filla et al., 1998; Christian et al., 2000; Park et al., 2003; Carrasco et al., 2005; Qu et al., 2010; Dejima et al., 2011; Palma et al., 2011). In FGF signaling, HSPGs stabilize the initial low-affinity complex of 1:1 FGF:FGFR. This ternary complex then leads to the dimerization of receptors, and subsequent activation of signaling pathway (Ornitz, 2000).

The members of the two main groups of HSPGs, syndecans and perlecans, are widely expressed in the developing embryo (Yamaguchi, 2001). In the developing brain, syndecan-1 and 4 localize in the ventricular zone (VZ), whereas glypican-4 is expressed both in the VZ and in postmitotic neurons (Ford-Perriss et al., 2003). In contrast, perlecan is localized exclusively in the basement membrane.

In the HSPG biosynthesis route, N-acetylglucosamine and glucuronic acid are added to the proteoglycan core protein (Ornitz, 2000). The synthesized heparan chains are then extensively modified to yield mature heparan sulphate molecules. These modifications, especially the pattern of O-sulphation, can be tissue-specific and thus provide an additional mechanism to regulate the binding of growth factors to their receptors (Shah et al., 2011).

1.1.2. Wnt signaling

Mammalian genome contains 19 genes encoding Wnt ligands, and 10 encoding Frizzled (Fzd) cell surface receptors. The binding of Wnts to their receptors induces a variety of responses in the cell. Traditionally, these responses have been divided into canonical, i.e. mediated by beta-catenin (Ctnnb1)/T-cell factor (TCF), and non-canonical responses. However, as Wnt activation often involves both canonical and non-canonical components, it has been suggested that Wnt signaling should be viewed as a single large network (van Amerongen and Nusse, 2009).

In the canonical pathway, the binding of Wnts to Fzds, and their interaction with low density receptor-related protein (LRP) leads, via Dishevelled, to the inactivation of ”destruction complex”, which consists of Adenomatosis polyposis coli (APC), Casein kinase I, Axin, and glycogen synthase kinase 3b. Without ligand binding, this complex phosphorylates beta-catenin, which leads to its ubiquination, and degradation in the proteasome. Inactivation of destruction complex allows beta-catenin to enter the nucleus, where it regulates transcription together with TCF/Lymphoid enhancer binding factor (LEF) transcription factors. Non-canonical, i.e beta-catenin independent responses, include at least three pathways involving Ca^{2+} as a second messenger, and a divergent canonical pathway which is involved in axon growth and synapse remodelling (Twyman, 2009).

These pathways can be modulated on s everal levels, including LRP availability, regulated by Dkk and Kremen and members of Sost family; Wnt-receptor complex

activity, via Norrin and R-spondin-2; presence of co-factors such as Cthrc1; and secreted inhibitors, such as Wifs and Sfrps (van Amerongen and Nusse, 2009; Twyman, 2009).

Wnt signaling is involved in numerous aspects of embryogenesis. It regulates stem cell maintenance, cell proliferation, movements and fate decisions, as well as the establishment of embryonic axes and tissue polarity (van Amerongen and Nusse, 2009). Compared to the canonical pathway, the non-canonical responses remain less characterized during early CNS development. The Wnt-responses involving Ca^{2+} have been identified during gastrulation, when they modulate cell movements. These responses act via activation of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) and phospholipase C, phospholipase C, or cytoskeleton movements via JNK.

1.1.3. Shh signaling

Shh belongs to the Hedgehog family, which in vertebrates consists of three members – in addition of Shh, also Indian hedgehog and Desert hedgehog have been identified. Two transmembrane proteins, Patched1 (Ptc1) and Smoothened (Smo) mediate the Shh signal transduction. In the absence of Shh, Ptc1 inhibits Smo activity and translocation to the primary cilium (Ribes and Briscoe, 2009). In these cells, protein kinase A is active and promotes cleaving or complete degradation of Gli1-3 transcription factors. The cleaved Gli2R and Gli3R proteins act as repressors in the nucleus, preventing the expression of Shh target genes (Litingtung and Chiang, 2000). Binding of Shh to Ptc1 relieves the inhibition of Smo, which moves into the primary cilium. Smo prevents the function of protein kinase A, and this results in the presence of more Gli activators leading to the transcription of Shh target genes.

Several feedback modulators of Shh pathway have been identified. Cell surface proteins Gas1, Cdo and Boc potentiate Shh signaling, likely by introducing Shh to Ptc1 (Ribes and Briscoe, 2009). These factors are repressed by Shh signaling. In contrast, Shh signaling upregulates negative feedback regulators of the pathway, Ptc1 and Hhip1. These feedback loops modify the extent of morphogen activity, thus providing precision to pattern formation.

During embryonic development, Shh signaling is involved in several processes, including patterning of the limb bud, ventralization of the neural tube, and specification of neuronal fates.

1.1.4. TGF-beta signaling

The large superfamily of TGF-betas consists of three subfamilies: BMPs, TGF-betas, and activin/inhibins. They bind to heteromultimeric serine/threonine kinase receptors, which contain subtype I and type II receptors (Chen et al., 2004). Upon ligand binding, type II receptors phosphorylate, and thus activate, the kinase in type I receptors. Type I receptors then activate downstream targets, such as SMADs, which alter gene expression. In addition, they are believed to activate several other kinase pathways including MAPK, P13 kinase and PKC (de Caestecker, 2004).

TGF-beta signaling can be modulated on several levels. These include accessory receptors, endocytic trafficking of activated receptors, and ligand inhibitors. Of these, best characterized are cystein-rich extracellular proteins noggin and chordin, which inhibit BMP-receptor-interaction by directly binding to ligands (Kishigami and

Mishina, 2005). Intracellular inhibitors include Tob1 and Smurf1 (Chen et al., 2004). The most important inhibitor of activins is follistatin, which blocks their function by binding them with high affinity (Nakamura et al., 1990).

The many functions of TGF-beta-mediated signaling, especially BMPs, include the formation of the primitive streak and regulation of gastrulation, specification of embryonic axes, and organogenesis (Kishigami and Mishina, 2005). BMPs are known dorsalizing factors, which antagonize Shh-mediated ventralization. In the nervous system development, inhibition of BMPs is required for the formation of neural tissue.

1.1.5. Retinoic acid signaling

Retinoic acid is a lipid which is synthesized from vitamin A (retinol). The synthesis occurs in two steps: first retinol is reversibly oxidized into retinaldehyde, which is then irreversibly oxidized into all-trans retinoic acid (Duester, 2009). Several alcohol dehydrogenases and shortchain dehydrogenase/reductases are involved in the first oxidization step. The second step involves only three members of retinaldehyde dehydroxylases: Aldh1a1, Aldh1a2, and Aldh1a3 (also called Raldh1, Raldh2, Raldh3). In the developing embryo, these three enzymes are tissue-specific and they are expressed in non-overlapping patterns.

Cellular retinoic-acid binding proteins (CRABP) 1 and 2 bind to the newly synthezised retinoic acid in many tissues (Maden, 2007). CRABP2 escorts cytoplasmic retinoic acid into the nucleus. There retinoic acid binds to a transcription factor complex, which is a heterodimer of retinoic acid receptor (RAR) and a retinoic X receptor (RXR). This complex then recognizes and binds to a retinoic-acid response element (RARE) in DNA. Although RARE has been found only in 27 genes, several hundred genes are known to be retinoic acid responsive, suggesting a RARE-independent mode of action (Maden, 2007). P450 family of enzymes degrade retinoic acid in the cytoplasm, and their activity also limits the distribution of retinoic acid from the synthesis site.

In the developing CNS, retinoic acid is involved in both D-V and A-P patterning, as well as inducing neuronal differentiation, especially in the caudal parts.

1.1.6. Notch signaling

The above mentioned signaling systems rely on secreted ligands and can thus operate on both short and long distance. In contrast, in Notch pathway both ligands and receptors are transmembrane proteins, which restricts the range of Notch signaling between neighboring cells (**Figure 2**).

Mature Notch receptor (Notch1-4 in mammals) consists of an extracellular part, a single-pass transmembrane region, and a small intracellular domain (NICD) (Kovall and Blacklow, 2010). Notch ligands require endocytic processing to become functional and gain ability to bind Notch. In mammals, the ligands are either Serrate-like (Serrate-1 and 2, usually called Jagged-1 and 2) or Delta-like (Dll1, 3, and 4), named after their *Drosophila* homologues. Receptor-ligand interaction induces a series of proteolytic cleavages in the extracellular and transmembrane parts of Notch. This releases the NICD, which is transported into nucleus. There NICD interacts with DNA-binding Rbpj protein and several co-activatiors, such as Mastermind, to activate transcription. In the absence of Notch, Rbpj recruits co-repressors and prevents the expression of Notch targets (Miyamoto and Weinmaster, 2009). Other components of Notch signaling are

Neuralized-like (Neurl) and Mindbomb (Mib), which ubiquinylate Notch ligands, and Numb which in *Drosophila* inhibits Notch signaling. However, in vertebrates the role of Numb appears to be more complicated (see "1.2.3.2. Apico-basal polarity of neuronal progenitors").

Well-known Notch targets are the members of *Hairy/Enhancer(Split)* family *(Hes)*, six of which (*Hes1-3, 5-7*) are expressed in mouse (Kageyama et al., 2007). Together with co-repressors, Hes factors function as homo- and heterodimers to repress the expression of Notch target genes, such as proneural genes and Notch ligands. In the developing CNS, especially Hes1 and Hes5 appear to be essential effectors of Notch signaling (Ohtsuka et al., 1999; Ohtsuka et al., 2001; Hatakeyama et al., 2004). They can inhibit their targets by binding them and thus preventing their access to DNA, or by repressing their transcription. In the neighboring cell with less or no Notch-activity, these proneural genes can be expressed and positively autoregulate themselves. This lateral inhibition regulates the timing of neurogenesis, neuronal fate, or both, between neighboring cells (see "1.2.3.7. Lateral inhibition").

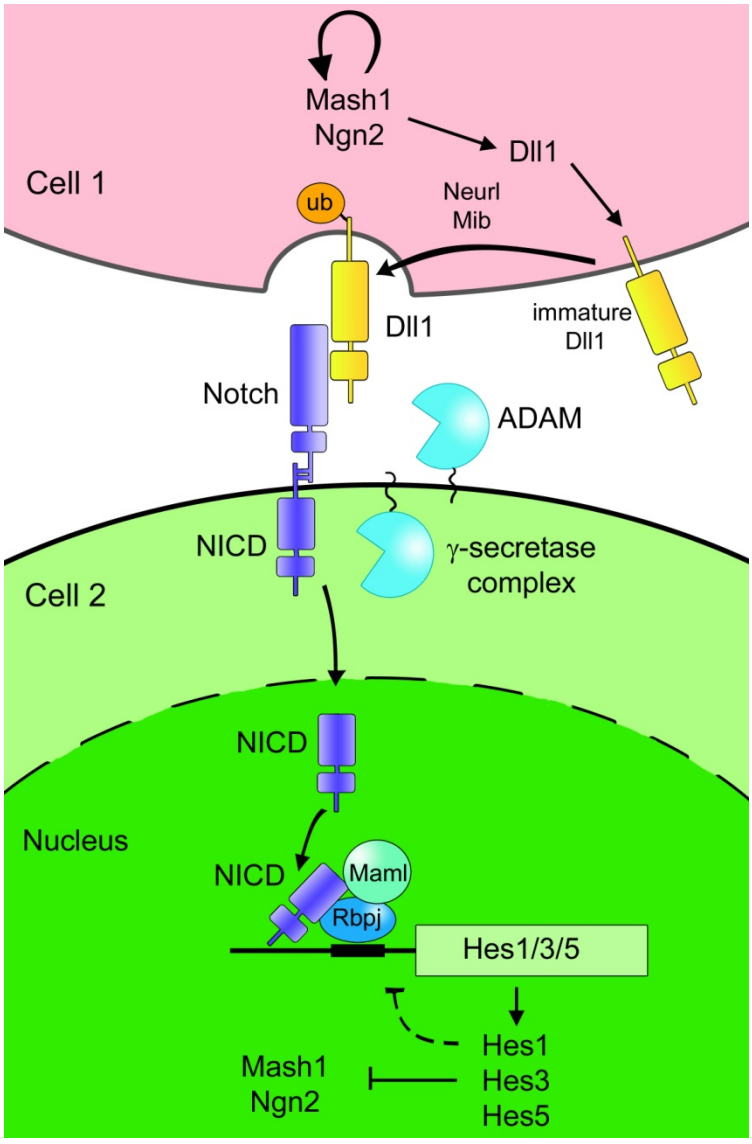


Figure 2. Notch signaling pathway and lateral inhibition. A simplified scheme on Notch signaling pathway between two cells. In the neurogenic cell (1) proneural factors induce the expression of Notch ligands, such as *Dll1*. The immature ligands need to be processed and ubiquinylated before they can activate Notch receptors. Membrane-bound proteases ADAM and gamma-secretase complex release Notch intracellular domain (NICD), which moves into the nucleus. There NICD activates transcription of target genes together with co-factors, such as Rbpj and Mastermind-like (Maml). Common Notch-targets are Hes-family of transcription factors, which repress both transcription and function of proneural genes. This prevents neuronal differentiation in Notch-expressing cell (2). Hes1 can also repress its own expression. ub, ubiquitin. Based on Kageyama et al., (2008).

1.2. Early development of the CNS

1.2.1. Neural induction

The development of the CNS – the brain and the spinal cord – begins in the process of neural induction. As a consequence, the induced ectoderm adopts neural identity, whereas the rest of the ectoderm becomes epidermis, forming skin and its appendages. The concept of neural induction was discovered in the studies by Spemann and Mangold, in which transplantation of the amphibian blastopore generated a second axis in the host embryo. Later studies revealed that the mechanisms of induction were highly conserved between different vertebrate species, which all had a similar organizer tissue, termed Hensen's node in birds and node in mammals (reviewed in Nieto, 1999; Viebahn, 2001). As the organizer cannot be clearly defined before the onset of gastrulation, the first steps of neural induction were thought to occur at the formation of primitive streak and the beginning of gastrulation. However, the expression of pre-neural genes before these stages suggests that neural induction might begin already earlier (see below).

Experiments with dissociated animal caps of frog embryos, and identification of several BMP inhibitors in the organizer, such as noggin and chordin, lead to the classic neural default model. It states that neural tissue is the default state of ectoderm, and that other ectodermal cell types are actively induced (Hemmati-Brivanlou and Melton, 1997a,b; Levine and Brivanlou, 2007).

The complete absence of ectoderm inducers, especially BMPs, would allow cells to adopt neural fate, whereas intermediate and high BMP levels would lead to the development of the border between neural tissue and epidermis, and epidermis, respectively. In addition to BMPs, the inducers of non-neural fate include Nodal and Wnts (Tam, 2004). This default model has gained further support from experiments with other model organisms and embryonic stem cells (Munoz-Sanjuan and Brivanlou, 2002).

However, other studies, especially in chick embryos, have revealed that neural induction is a much more complex process, which has challenged the inhibition-based default model. The evidence against the default model suggests that the inhibition of BMPs alone is not sufficient to produce neural tissue (Stern, 2005). Furthermore, ectoderm expresses early pre-neural genes *SRY-box containing gene (Sox) 3* and *Early response to neural induction (Erni)*, before the onset of gastrulation (Streit et al., 2000; Wilson et al., 2000). Thus, the earliest events of neural induction may begin before the onset of gastrulation, and before the formation of a clear organizer. The main signal to induce the expression of these early pre-neural genes is thought to be FGF8 from the underlying endoderm (hypoblast in chick, visceral endoderm in mouse), although in the epiblast itself, some FGF3 is expressed (Wilson et al., 2000, Streit et al., 2000; Knezevic and Mackem, 2001). Later, FGF-induced Churchill stops cell ingression via Sip1 (Sheng et al., 2003). The epiblast cells which stay on the surface, become sensitized to BMP inhibitors and other signals, and are then able to commit to neural fate. This way, the neural induction comprises of two processes: the choice between neural tissue and epidermis, and the establishment of the boundary between neural plate and the ingressing cells which will form mesoderm (Sheng et al., 2003). However, the exact pathway from FGFs to the induction of definitive neural marker *Sox2* in the neural plate remains unclear.

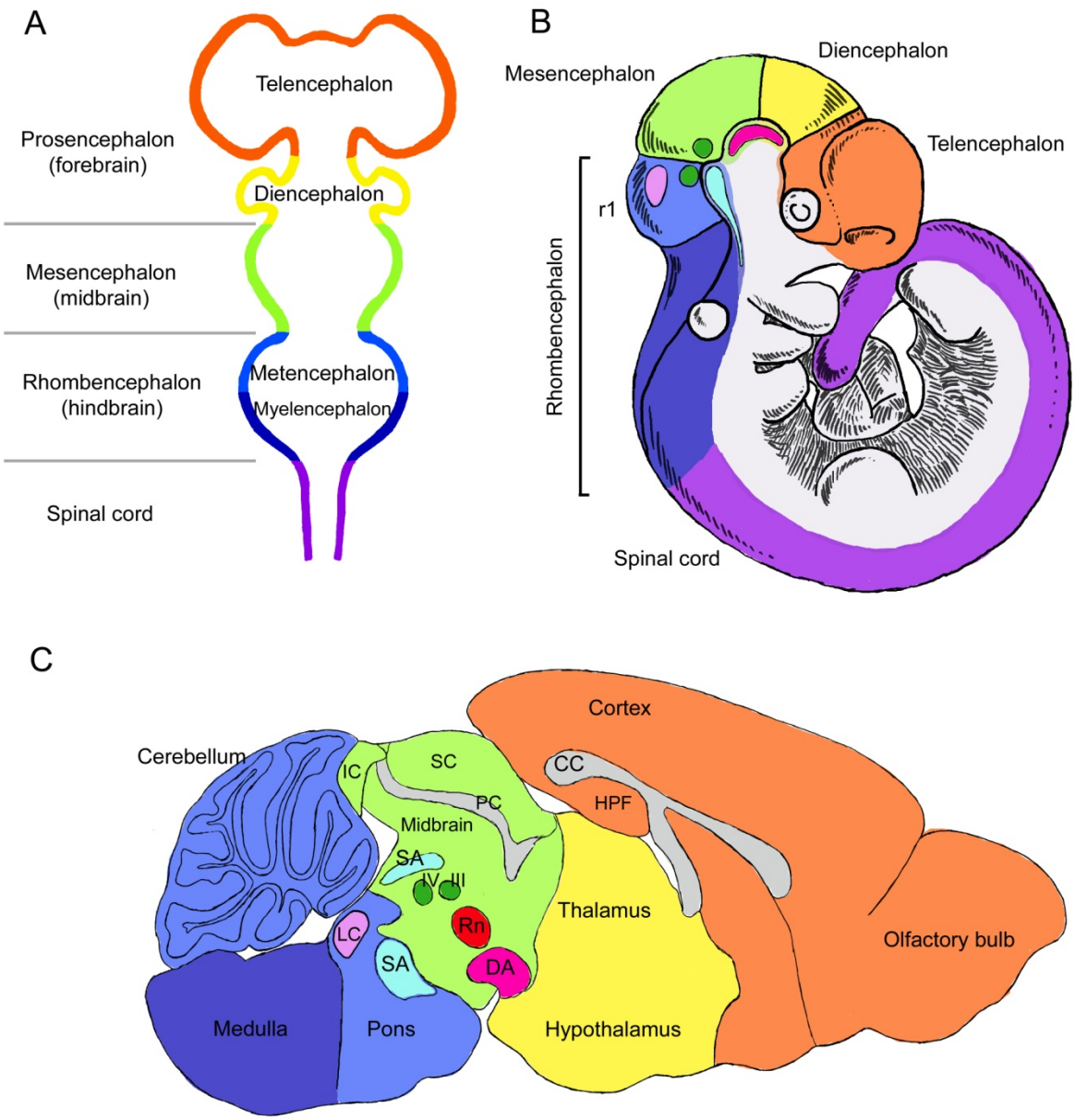


Figure 3. Brain development in mammals.

(**A**) Anterior part of the neural tube consists of vesicle-like structures, which will form the brain: prosencephalon, which is further divided into telencephalon and diencephalon; mesencephalon; and rhombencephalon which consists of anterior metencephalon and caudal myelencephalon. The rest of the neural tube will form the spinal cord. (**B**) Schematic view on embryonic day 10.5 mouse, showing different colour-coded regions of the CNS. Hindbrain is further divided into segmental units, rhombomeres. In this view, the most anterior part of metencephalon - rhombomere 1 - is highlighted with light blue, whereas the rest of the hindbrain is darker blue. Metencephalon includes also rhombomeres 2 and 3. Several important nuclei develop in the midbrain and hindbrain: dopaminergic neurons (pink), serotonergic neurons (turquoise), III and IV cranial nerves (dark green) and locus coeruleus (light purple). (**C**) Sagittal view of adult mouse brain, with color-coded brain regions showing derivatives of embryonic brain regions from (A). In the adult brain, some serotonergic neurons are also located in the midbrain. CC, corpus callosum; HPF; hippocampal formation; DA; dopaminergic neurons; LC, locus coeruleus; r1, rhombomere 1; Rn, red nucleus; SA; serotonergic neurons; III, third cranial ganglion (oculomotor complex); IV, fourth cranial ganglion (trochlear nucleus); IC, inferior colliculus; SC, superior colliculus; PC, posterior commissure. The adult brain was redrawn and modified from The Allen Brain Atlas.

In summary, the contribution of different molecules, such as BMP inhibitors, Wnt inhibitors, FGFs, and yet unknown molecules to the neural induction is a multistep process (Dorey and Amaya, 2010; Stern, 2005; Wills, 2010). The contradictory results may stem from differences between experimental approaches and model species. It has also been suggested that signals regulating neural induction might originate from several organizers in different parts of the axis. This hypothesis, originally presented by Mangold in 1930s (reviewed in Stern, 2005), is supported by the observations that many regions which adopt neural fate are never close to the organizer and that node-defective *FoxA2* and *cripto* mutants are able to develop nervous tissue (Ang and Rossant, 1994; Weinstein et al., 1994; Liguori et al., 2003). Anterior visceral endoderm (AVE), which is formed in these mutants, has been suggested to be a "head organizer".

1.2.2. Neurulation

After the formation of the neural plate, it begins to roll into a neural tube, in a process of neurulation. Brain and the anterior regions of the spinal cord form via primary neurulation. The plate first elongates and narrows by convergent extension movements, directed proliferation, and apico-basal cell elongation. Then the edges of the plate begin to rise up, forming a neural groove, and fuse together to form the neural tube. In mouse, the neural tube closure begins at the hindbrain/cervical junction at embryonic day (E) 8.5, and continues both anteriorly and posteriorly (Copp et al., 2003). Finally, the anterior and posterior openings of the neural tube, termed neuropores, are closed by E9.0. During this process, the anterior part of the tube bends and constricts in several places, forming three vesicle-like structures: prosencephalon, mesencephalon and rhombencephalon (**Figure 3A**). Rhombencephalon is further divided to anterior metencephalon and posterior myelencephalon, and prosencephelon into anterior telencephalon and caudal diencephalon. These structures will give rise to forebrain, midbrain and hindbrain, which will develop into the adult brain structures, including cortex, basal ganglia, thalamus, brain stem, and cerebellum (**Figure 3B,C**). More caudal regions of the neural tube will form the spinal cord. The most caudal part of the spinal cord forms via secondary neurulation. This is characterized by the formation of a cell cluster, called medullary cord, from the tail bud. Within the cluster, several lumens form, which then fuse together.

1.2.3. Self-renewal and differentiation of neuronal progenitors

After the neural tube closure, and formation of the initial layout for CNS, the future brain consists of a single cell layer of neuroepithelial cells lining the future brain ventricles. In order to form the complex network of neurons in the adult brain, these cells need to both proliferate extensively, and then produce the required number of each type of neurons in a coordinated fashion.

The neuroepithelial cells are able to both self-renew, and to produce all the neuron and glial cells in the adult brain – i.e they are multipotent. Hence, these cells are often called neuronal stem cells. At the onset of neurogenesis, more committed but still proliferative cell types, such as radial glial cells, emerge (see 1.2.3.1. "Structure of neuroepithelium, and characteristics of different neuronal progenitor types"). Together, these proliferating cells are called neuronal progenitors.

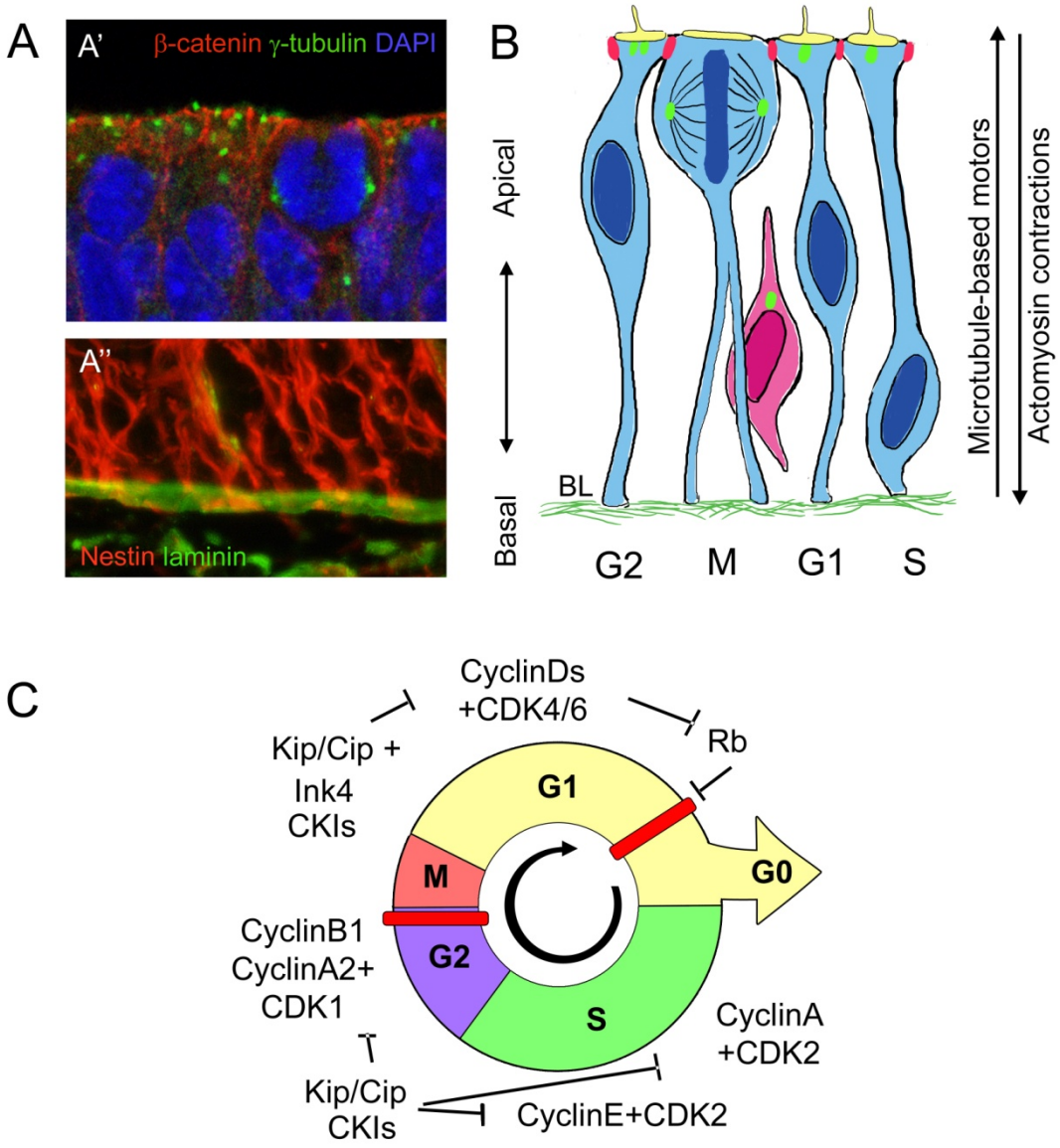


Figure 4. Structure of neuroepithelium, and proliferation of neuronal progenitors.
(**A**) Confocal images of embryonic midbrain VZ stained with antibodies. (**A'**) Beta-catenin (red) stains adherens junctions and basolateral membranes. Gamma-tubulin (green) is located in the centrioles, which regulate spindle orientation during mitosis. One cell is undergoing mitosis near the apical membrane. DNA i s visualized with DAPI (blue). (**A''**) Basal side of neuroepithelium, showing basal lamina (green) and basal processes of neuronal progenitors (red). (**B**) Schematic view on interkinetic nuclear migration. Elongated neuronal progenitors contact both apical and basal sides of neuroepithelium, and their nuclei migrate up and down according to cell cycle progression. DNA duplicates in the basal side, whereas mitoses occur apically. This movement is powered by micro-tubule-based motors and actomyosin contractions. When a p rogenitor exits the cell cycle (pink), it detaches from basal lamina and other progenitors, and moves to MZ. Yellow structures depict apical membrane domain which contains a p rimary cilium. Centrioles (green) are located under the cilium. (**C**) Cell cycle. Cyclins and CDKs drive the cycle forward, whereas CKIs inhibit it. Two major check-points (red) take place before S-phase and mitosis. Phosphorylation state of Rb regulates the entry into S-phase. BL, basal lamina.

After the initial expansion phase of repeated proliferative divisions, some neuronal progenitor divisions begin to produce daughter cells which exit the cell cycle and become post-mitotic neuronal precursors. This is called neurogenic period (Temple, 2001; Hirabayashi and Gotoh, 2005), and in mouse it begins around E10.5. When neurogenesis begins, progenitors exit the cell cycle and begin to express genes typical of differentiating neurons, such as *Tuj1* and *HuC/D*. The postmitotic precursors detach from the neuroepithelial layer and begin to migrate away from it. Thus, neurogenesis turns the single-layered neuroepithelium into a multilayered structure. The layer which contains progenitors and faces the lumen of the ventricle is then called the VZ. The newly post-mitotic cells form an intermediate zone (IZ) between the VZ and the postmitotic layer(s). Postmitotic cells are found in the mantle zone (MZ), which in the forebrain is organized into six layers formed in an inside-out fashion. Elsewhere in the CNS, MZ consists of a single layer. The most outer region of the MZ – marginal zone – consists mostly of fibers and will form the white matter, whereas the rest of the MZ will form the grey matter.

After the neurogenic phase is over, gliogenic phase begins. During this time, distinct precursor cells give rise to oligodendrocytes, astrocytes, and ependymal cells (Rowitch and Kriegstein, 2010). Radial glia can also directly transdifferentiate into astrocytes. In order to produce all the required neuronal and glial cell types in correct amount, neurogenesis must be tightly regulated. Too rapid neurogenesis will deplete the progenitor pool, whereas overproliferation may result in tumors. In both cases, disrupted balance between self-renewal and differentiation results in abnormal brain structure and function.

In the following, I will briefly describe the structure of the VZ, regulation of cell cycle, and properties of neuronal progenitors. Emphasis will be on factors which affect proliferation vs differentiation decisions.

1.2.3.1. Structure of neuroepithelium, and characteristics of different neuronal progenitor types

Neuroepithelial cells have highly elongated cell morphology, and they contact both sides of the epithelium: a small apical side process abuts the ventricle, and a long basal process extends to the basal lamina (**Figure 4A**). The nuclei of these tightly packed cells migrate up and down along the apico-basal axis during the cell cycle, creating a pseudostratified structure (see below). The apical process contains a primary cilium, which is thought to be involved in signal transduction. A small membrane domain of the basal process contacts the extracellular matrix in the basal lamina via integrins. The basal process can function as a guide, along which the developing neurons can migrate away from the VZ (Rakic, 2003).

Neuroepithelial cells are able to both self-renew, and to produce daughter cells which have properties of both astroglial and neuroepithelial cells (Malatesta et al., 2000; Götz and Huttner, 2005; Pinto and Götz, 2007; Rowitch and Kriegstein, 2010). These more committed cells, called radial glia, appear at the onset of neurogenesis (**Figure 5**). They are also highly elongated, contact both sides of the neuroepithelium, and have a capacity for self-renewal. In addition, they are also able to produce astrocytes, neurons, and oligodendrocytes. However, patterning signals in the CNS, such as FGFs, Shh, and BMPs, restrict the differentiation potential of radial glia in different regions (Rowitch and Kriegstein, 2010). These restrictions appear to increase as the embryogenesis

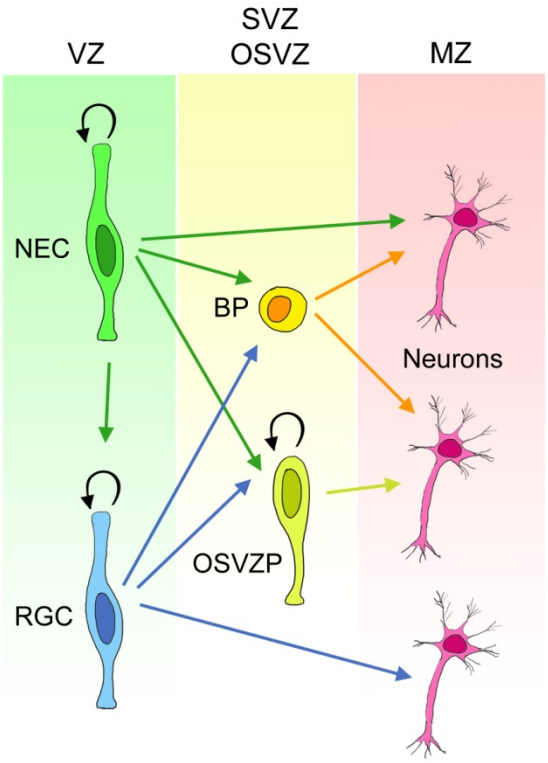


Figure 5. Neuronal progenitor types in the brain.
In the VZ, neuroepithelial cells (NEC) can both self-renew and produce more committed progenitor cells, radial glial cells (RGC). In the forebrain, both NEC and RGC give rise to basal progenitors (BP), located in the SVZ, and outer subventricular zone progenitors (OSVZP). Basal progenitors can only divide symmetrically to produce two neurons. OSVZPs are able to self-renew and to produce neurons. Neurons are located in the MZ, which in the forebrain is further divided into layers. After the production of neurons, radial glial cells produce oligodendrocytes and then astrocytes (not shown).

proceeds (Götz and Huttner, 2005). In fact, retrovirally-mediated lineage analysis, FACS sorting of GFP-labeled VZ cells, and live imaging of dividing radial glial cells have shown that most radial glial cells give rise to only one cell type – neurons or glia (Pinto and Götz, 2007). However, a small subset of radial glia retains capacity to generate multiple cell types. Gradually radial glial cells replace the original neuroepithelial cells, and in fact most of the neurons and macroglia in the adult brain are derived from these cells.

Radial glia maintain the expression of several neuroepithelial markers such as nestin, but they also express several genes not found in neuroepithelial cells, and which are more typical of astroglial cells (Götz and Huttner, 2005; Pinto and Götz, 2007). These include astrocyte-specific glutamate transporter (GLAST), glial fibrillary acidic protein (GFAP), vimentin, and brain-lipid-binding protein (BLBP). In addition, radial glia contain glycogen granules, which are not present in neuroepithelial cells. Despite all these molecular characteristics, which are found in radial glial but not neuroepithelial cells, drawing the line between these two cell types has proven difficult, especially *in vivo*. Thus, these cells are often grouped under a common term – neuronal progenitor cells.

In telencephalon, additional types of non-VZ neuronal progenitors exist. Basal progenitors are derived from both neuroepithelial cells and radial glial cells, but have retracted their apical and basal contacts and migrated away from the VZ (Miyata et al., 2004). In the SVZ, basal progenitors, which are unable to self-renew, then produce two neurons in each cell division. They express several genes not found in VZ progenitors, such as *Tbr2*, *Cux1*, *Cux2*, and *Vglut2*, and also lack expression of *Hes* factors and *Pax6* (Pinto and Götz, 2007). Furthermore, a new type of progenitors was recently identified in the developing cortex of human, ferret, and mouse (Hansen et al., 2010; Fietz et al., 2010; Shitamukai et al., 2011; Wang et al., 2011). In contrast to basal progenitors, these

outer subventricular zone (OSVZ) progenitors retain a radial-glia-like morphology, and they contact basal lamina but not the apical surface. Furthermore, these progenitors express *Sox2*, *Pax6*, and *Hes1*, like neuronal progenitors in the VZ. OSVZ progenitors are able to both self-renew and generate neurons (**Figure 5**).

In the following chapters, the term "neuronal progenitors" will refer to proliferative cells – both radial glia and neuroepithelial cells – located in the VZ. Basal progenitors and OSVZ progenitors are named accordingly. The term "neuronal precursors" refers to postmitotic cells which have not yet undergone terminal differentiation to become mature neurons.

1.2.3.2. Apico-basal polarity of neuronal progenitors

In the apical side, neuronal progenitors contact each other mainly via cadherin-based junctional complexes (Aaku-Saraste et al., 1996; see also **Figure 4A'**). In addition, neuroepithelial cells, unlike radial glial cells, contain functional tight junctions. Whereas the extracellular domain of cadherins mediates cell-adhesion via homophilic interactions, the cytoplasmic domain interacts with catenins (Stepniak et al., 2009). Together with actin filaments, these junctional proteins form ring-like structures around the apical membrane domain. These contact points do not only function in cell adhesion. Cadherins and catenins have been shown to interact with several major signaling pathways, and thus disruption of adherens junctions may either upregulate or downregulate these signals (Stepniak et al., 2009). For example, phosphorylated tyrosine is localized in these structures in later embryonic stages (Chenn et al., 1998).

More importantly, beta-catenin, a structural protein but also a component of Wnt-signaling pathway, is localized around the apical domain of progenitor cells (Farkas and Huttner, 2008; see also **Figure 4A'**). Beta-catenin-mediated signaling promotes proliferation of neuronal progenitors, and its disruption leads to both premature neurogenesis and the loss of apico-basal polarity, whereas overexpression expands the progenitor pool (Machon et al., 2003; Chenn and Walsh, 2003; Zechner et al., 2003; Chilov et al., 2010; Chilov et al., 2011).

The adherens junctions separate two membrane domains in each cell: a small apical domain, and the larger basolateral membrane (Kosodo et al., 2004). The apical domain contains specific transmembrane proteins, such as Prominin-1 (CD133), as well as proteins associated with centrosomes, such as gamma-tubulin (Aaku-Saraste et al., 1996; Weigmann et al., 1997; Chenn et al., 1998). In addition, the basal lamina - contacting part of the basal process appears to form a specific membrane domain. Thus, neuronal progenitors have an apico-basal polarity.

Several protein complexes in both apical and basolateral surfaces maintain epithelial polarity (Margolis and Borg, 2005). In neuronal progenitors, a central polarity-regulator complex in the apical side includes atypical protein kinase C lambda (aPKCλ), Par3, and Par6. Together they coordinate the functions of several polarity regulating proteins, such as Cdc42/Rac1 GTPases. Other polarity complexes include PALS1/MALS (also known as Veli), and PALS1/CRB3/PATJ (Margolis and Borg, 2005).

Inactivation of adherens junctions or polarity regulators produces various phenotypes. In some cases, progression of neurogenesis is not majorly altered and non-polarized progenitors are able to continue proliferating. For example, the inactivation of *Cdc42* or *aPKCλ* leads to loss of adherens junctions. This results in an disordered VZ, where progenitors "leak" into the ventricle and mitoses occur in ectopic locations, but the

progression of neurogenesis remains relatively unaffected (Cappello et al., 2006; Imai et al., 2006). Loss of *N-cadherin* in the cerebral cortex similarly disrupts the cortical structure, but in this study the cell cycle progression was not analyzed (Kadowaki et al., 2007).

In some cases, the loss of polarity regulators does affect the cell cycle progression and neuronal differentiation. Ablation of polarity regulator *MALS* results in premature neuronal differentiation, whereas inactivation of small Rho GTPase *RhoA* leads to increased cell proliferation (Srinivasan et al., 2008; Katayama et al., 2011). The latter appears to result from increased Shh signaling. These different effects may result from partial functional redundancy of the proteins, or timing of inactivation. Apicobasal polarity might have a more prominent role during early brain development. Indeed, apical polarity complex is gradually downregulated during embryogenesis (Costa et al., 2008).

Another critical component of the polarity-regulating machinery is Numb. In *Drosophila*, Numb functions as a neuronal cell fate determinant, which asymmetrically segregates into the basal daughter cell and inhibits Notch signaling (Doe et al., 1998). This prompted speculation that the distribution of vertebrate homologues of Numb might similarly regulate the fate of daughter cells (Cayouette and Raff, 2002; Johnson, 2003; Shen et al., 2002). In vertebrate neuronal progenitors, Numb (*mNumb* and *Numbl* in mammals) is localized in vesicles near the adherens junctions, where it interacts with cadherins (Rašin et al., 2007). However, the loss of Numb does not lead to increased Notch signaling, as one might expect on the basis of *Drosophila* studies. In fact, the phenotype of *mNumb* and *Numbl* mutant embryos resembles greatly that of Notch-pathway mutants – neuronal progenitors begin to differentiate prematurely (Petersen et al., 2002; Petersen et al., 2004; Petersen et al., 2006). Numb also maintains the structure of the neuroepithelium, as conditional inactivation of *mNumb* and *Numbl* in radial glia leads to loss of polarity and ectopic mitoses (Rašin et al., 2007).

1.2.3.3. Basal process and basal lamina

Basal lamina is a thin layer of extracellular matrix, which is composed of laminins, collagen IV, nidogen, and HSPGs, for example perlecan and agrin (Erickson and Couchman, 2010; Timpl, 1996; Paulson, 1992), and is enriched with various growth factors (Colognato and ffrench-Constant, 2004). The basal process of the neuronal progenitors contacts the basal lamina via integrins, such as alpha-6, alpha-7, and beta-1 which are expressed already in E10.5 cortex (Lathia et al., 2007).

In addition, the contact points between basal process and basal lamina include growth factor receptors, and cross-talk between integrins and growth factor receptors can intensify signaling (Colognato and ffrench-Constant, 2004). Integrins may increase phosphorylation and expression levels of growth factor receptors, which in turn can promote the function of integrins. For example, the maintenance of neurospheres, derived from postnatal rats and mice, requires beta1-integrin-mediated activation of MAPK signaling (Campos et al., 2004), and basal lamina contacts can alter growth factor -mediated signaling to oligodendrocytes (Colognato et al., 2002).

It was long believed that during mitosis, a progenitor retracts its basal process and extends it back towards basal lamina after the cell division. However, several observations from different systems have shown that many neuronal progenitors retain basal process during mitosis (Miyata et al., 2001; Cayoette and Raff, 2003; Das et al.,

2003; Miyata et al., 2004; Afonso and Henrique, 2006). Remarkably, it was shown that the basal process can be divided during cell division and distributed between daughter cells (Kosodo et al., 2008). It is yet unclear whether this occurs in all neuronal progenitors. Similarly, it has been controversial whether the daughter cell which asymmetrically inherits the basal process stays as a progenitor or exits the cell cycle (Kosodo and Huttner, 2009).

Recently it was observed that upon neuronal progenitor divison, the daughter cell requires both apical components and the basal process in order to stay as a proliferative progenitor (Konno et al., 2008). Retaining only the apical components, but losing the basal process, is not enough to maintain the progenitors in a proliferative state, but instead leads to exit from the cell cycle. Together, these observations support idea presented already earlier that progenitors might receive signals via their basal lamina contacts (Miyata et al., 2001; Fishell and Kriegstein, 2003). These signals could affect the cell's survival, or decisions to proliferate or to differentiate. Indeed, radial glia cells in the cortex which lost contacts with the basal lamina, either due to loss of *integrin beta-1*, or *laminin alpha-2* and *alpha-4*, died apoptotically, which resulted in a decreased cortical size (Radakovits et al., 2009). Supporting this theory, the OSVZ progenitors, which also retain a connection to the basal lamina, remain proliferative (Hansen et al., 2010; Fietz et al., 2010; Shitamukai et al., 2011; Wang et al., 2011).

Surprisingly, when analyzing *perlecan*$^{-/-}$, *integrin gamma 1 I II4* $^{-/-}$, and *alpha 6 integrin*$^{-/-}$ mouse embryos in which neuronal progenitors lose their basal lamina contacts, Götz and colleagues did not observe any apparent defects in neurogenesis, interkinetic nuclear migration, or cell proliferation, although all mutants had a smaller forebrain (Haubst et al., 2006). *Integrin gamma 1 I II4* $^{-/-}$ embryos displayed only abnormal neuronal subtype composition and laminar organization, and only in late developmental stages. However, a more detailed analysis of *perlecan* mutants did reveal cell proliferation and neurogenesis defects in the forebrain, likely due to insufficient growth factor signaling (Girós et al., 2007). The phenotype became more severe with advancing corticogenesis.

Taken together, these findings indicate that neuronal progenitors in the VZ require both apical and basal processes to remain proliferative, buth that the function of these two domains is likely different. The main function of apical junctions appears to be maintaining the progenitors in the VZ, although they might also regulate various signaling pathways. In turn, the basal process might provide the progenitors with basal lamina -derived signals which prevent premature neurogenesis and support proliferation.

1.2.3.4. Interkinetic nucler migration

As mentioned above, nuclei of neuronal progenitors move along the apico-basal axis according to the phase in their cell cycle, in a process called interkinetic nuclear migration (**Figure 4B**). DNA duplication in the S-phase takes place near the basal side, from where the nuclei travel towards the apical side during G2. Mitosis (M-phase) occurs in the apical side, and then the nuclei of the daughter cells migrate back to the basal side during G1 (Baye and Link, 2008; Miyata, 2008; Taverna and Huttner, 2010). The suggested main purpose of interkinetic nuclear migration is to maximize the number of mitoses, but it might also control the exposure of the nuclei to environmental cues, which regulate proliferation versus differentiation decisions. One of these cues is might be Notch-signaling (Murciano et al., 2002).

Dynein and a member of kinesin-3 family, Kif1a, regulate the nuclear migration from basal to apical side (Tsai et al., 2010). Basal-to-apical movement also requires centrosomal proteins Cep120, TACCs and Hook3, which might reflect their function in microtubule organization (Ge et al., 2010; Xie et al., 2007; Taverna and Huttner, 2010). In addition, Lis1 regulates dynein, and reduction of Lis1 level leads to nuclear migration defects and ectopic mitoses (Gambello et al., 2003; Tsai et al., 2005). Similarly, mutations in other dynein-interacting proteins results in migration defects (Taverna and Huttner, 2010).

In contrast, it appears that apical-to-basal interkinetic nuclear migration depends on actomyosin contractility (Schenk et al., 2009). It is possible that in this direction, nucleus is not moved as a cargo but rather via directional myosin-II-dependent contractions. However, several observations suggest that microtubule-based kinesin motors may also regulate apical-to-basal nuclear movement (Taverna and Huttner, 2010). The individual contribution of these two systems: microtubule-based cellular motors and actomyosin contractions, on t he nuclear migration might depend on t he species and tissue type (Taverna and Huttner, 2010).

Inhibition of interkinetic nuclear migration using cytochalasin B does not prevent cell cycle progression, and results in ectopic mitoses (Götz and Huttner, 2005). The migration-defective progenitors also display increased neurogenesis, which might result from different spatial clues along the apico-basal axis (Murciano et al., 2002). Alternatively, cytochalasin B -treatment may result in the generation of basal progenitors, which could explain both increased neurogenesis and non-apically located mitoses (Taverna and Huttner, 2010).

However, progression of cell cycle is required for the nuclear migration, suggesting that master regulators of cell cycle also control the nuclear movement (Ueno et al., 2006). Factors required for the coupling of migration with cell cycle progression include Pax6 and microtubule-associated protein Tpx2 (Estivill-Torrus et al., 2002; Kosodo et al., 2011).

1.2.3.5. Cell cycle progression

Two most important cell cycle check-points take place before S-phase and mitosis (**Figure 4C**). These check-points ensure that DNA is correctly duplicated before mitosis, and that possible damage can be repaired before cell division. Loss of mitogenic or upregulation of neurogenic signals, or both, can induce the progenitor to exit the cell cycle before G1-S transition and enter G0 phase. This phase can be short-term or permanent, and appears to require active maintenance.

Research on cell cycle regulation in the nervous system has especially focused on the regulation of G1-S-phase transition (McClellan and Slack, 2006). Numerous proteins regulate this step, and one of the most studied ones is retinoblastoma (Rb) tumor suppressor. In its hypophosphorylated state, Rb inhibits the function of E2F transcription factors, thus preventing cell's entry into the S-phase (Sun et al., 2007). Conditional inactivation of *Rb* in the forebrain leads to increased proliferation of neuronal progenitors (Ferguson et al., 2002). A related family member, p107, regulates neuronal progenitor proliferation by mediating responsiveness to FGF2 (McClellan et al., 2009).

Activity of Rb is regulated at two levels: Cyclins and cyclin dependent kinases (CDKs) phosphorylate – and thus inactivate – it, while cyclin dependent kinase inhibitors (CKIs)

prevent the function of cyclin-CDK-complexes. During late G1, mitogenic signals induce CDK-Cyclin complexes to phosphorylate Rb, and it is kept in a phosphorylated state through the cell cycle until the early G1 of the next round (Sherr and Roberts, 1999). The loss of Rb-regulating Cyclins and CDKs leads to reduced proliferation of neuronal progenitors, and a subsequent decrease in the size of affected brain regions (McClellan and Slack, 2006).

CKIs prevent the progression of cell cycle by disturbing the function of Cyclin/CDK-complexes. They belong to two families: Ink4 which contains $p16^{Ink4a}$, $p15^{Ink4b}$, $p18^{Ink4c}$ and $p19^{Ink4d}$, and Kip/Cip which includes $p27^{Kip1}$, $p21^{Cip1}$, and $p57^{Kip2}$ (McClellan and Slack, 2006). Ink4 family specifically inhibits CyclinD/CDK4/6 complexes, while the activity of Kip/Cip family members is broader – they can inhibit also CyclinE/CyclinA/CDK2 complexes. Inactivation of CKIs leads to increased proliferation of progenitors, often resulting in apoptosis. In addition, the loss of N-Myc, which regulates the expression of several CKIs, CDKs and Cyclins, results in decreased proliferation, increased differentiation, and thus reduced brain mass (Knoepfler et al., 2002).

Not only the completion of the cell cycle, but also its length, appears to be important for the neural progenitors. Olomoucine, a chemically synthesized inhibitor of CDKs, slows down the cell cycle and triggers premature neurogenesis in telencephalon (Calegari and Huttner, 2003). Also *in vivo* a longer cell cycle has been associated with neurogenesis (Calegari et al., 2005). Especially the length of G1 appears critical - overexpression of CyclinD1/CDK4 inhibits neurogenesis by preventing lengthening of G1, while inactivation of CyclinD1/CDK4 has the opposite effect (Lange et al., 2009). Concomitantly, mitogens such as FGF2 accelerate G1 and increase self-renewing divisions, while neurotrophin 3 slows G1 causing differentiation (Lukaszewicz et al., 2002). Interestingly, a recent study by Huttner and colleagues shows that before entering neurogenesis, neuronal progenitors also shorten their S-phase (Arai et al., 2011).

According to the cell-cycle length hypothesis, postulated by Calegari and Huttner (2003), a longer cell cycle would allow more time for cell fate determinants to promote cell cycle exit. However, the exact nature of these determinants remains elusive.

1.2.3.6. Symmetric and asymmetric cell division

When neuronal progenitors divide, they produce two daughter cells which have either identical or different fates. In other words, a division can be symmetric or asymmetric (Götz and Huttner, 2005, Huttner and Kosodo, 2005). Symmetric divisions can produce two proliferative progenitors, or, for example in the case of basal progenitors, two neurons (**Figure 6**). Asymmetric divisions can produce a more committed progenitor type – for example, neuroepithelial cells can produce radial glial cells. Alternatively, asymmetric divisions can give rise to a neuronal or glial precursor which exits the cell cycle. Divisions which produce one or two neurons are also called neurogenic, and can be identified with *Tis21* expression (Farkas and Huttner, 2008).

In *Drosophila* nervous system, in which the events of neurogenesis were first characterized, symmetric cell divisions which occur perpendicular (vertically) towards the apical surface generate two proliferative neuronal progenitors (Betschinger and Knoblich, 2006). In contrast, horizontally occurring divisions are neurogenic: the larger apical daughter cell stays proliferative and the smaller basal daughter (ganglion mother

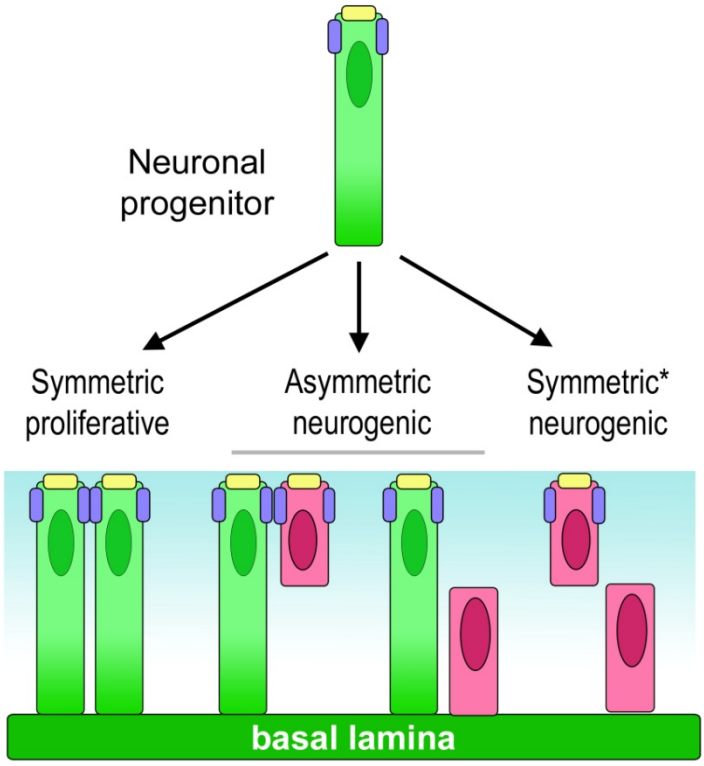


Figure 6. Symmetric and asymmetric divisions in the VZ.
When neuronal progenitor divides, it can either divide symmetrically to produce two daughter cells with same fate (either two progenitors or two neurons), or asymmetrically, to produce one progenitor and one neuron. Progenitors need both apical membrane components and a connection to basal lamina via basal process in order to stay as a proliferating progenitor (green). In a symmetric division, these structures are divided equally among daughter cells. If the daughter cell only inherits the basal process or the apical components in an asymmetric division, it will begin to differentiate (red). However, the connection between basal process inheritance and cell fate may be context dependent and other division types might exist. (*) Hypothetical model of symmetric neurogenic divisions in the VZ. This division type has been found in the SVZ, where non-polarized basal progenitors produce two neurons in each divisions. Based on Konno et al., (2008) and Kosodo and Huttner (2009).

cell) begins to differentiate as a neuron. The apical daughter inherits adherens junctions and apical polarity complex, as well as Notch signaling components.

In vertebrates, most neuronal progenitors divide in a vertical manner (Huttner and Kosodo, 2005; Kosodo and Huttner, 2009). During symmetric proliferative divisions, the apical membrane components and adjacent adherens junctions are thought to be bisected between the daughter cells. However, vertical divisions can also be asymmetric. Similarly to the situation in *Drosophila*, the inheritance of apical components – which in vertebrates include Prominin1-rich apical membrane domain and adherens junctions – has been suggested to play a role in the maintenance of proliferative status (Kosodo et al., 2004; Huttner and Kosodo, 2005). However, it is not the only factor in this process. Recent studies have revealed the importance of the basal process, which at least in some neuronal progenitors is split during symmetrical divisions (Konno et al., 2008; Kosodo et al., 2008). The fate of the daughter cell which

inherits the basal process has been a matter of controversy. Ogawa and colleagues (Miyata et al., 2001) suggest that asymmetrical inheritance of the process leads to cell cycle exit, whereas Matsutagi's team (Konno et al. 2008) show that at least in some progenitors, retaining both basal process and apical domain is required to maintain the proliferative status. It might be that these results stem from different experimental settings, or there are differences between neuronal progenitors in different areas, and different developmental stages.

As both basal process and apical membrane represent very small domains, their equal splitting between daughter cells requires tight control. Even a small deviance from a correct division plane may result in an unequal distribution of these components between daughter cells (Kosodo et al., 2004). At the time of mitosis, a mitotic spindle forms which then separates chromosomes into opposite poles of the dividing cell. Initially, the mitotic spindle oscillates in a seemingly random pattern until setting into the final angle (Heins et al., 2001). Several proteins such as LGN, ASG3, Numa, small heterotrimeric G proteins, and mINSC, help to orientate the spindle into the correct position together with apical polarity regulators (Buchman and Tsai, 2007). However, upstream signaling mechanisms which regulate spindle positioning remain unclear.

The division plane of cytoplasm follows the orientation of the mitotic spindle, starting in the basal side of the cell and continuing apically (Kosodo et al., 2004; Dubreuil et al., 2007; Kosodo et al., 2008). Thus, the orientation of the spindle appears to determine the cell cleavage plane. Indeed, spindle misorientation, for example by inactivating spindle-organizer *Aspm* via RNAi, has been shown to result in more asymmetric divisions, increasing neurogenesis (Haubensak et al., 2004; Fish et al., 2008). In contrast, *Aspm*$^{-/-}$ mutant mice do not display abnormal spindle positioning or increased neurogenesis (Pulvers et al., 2010). These contradicting results might be due to differences in the experimental setting or the presence of a partly functional protein in the *Aspm* mutants (Haubensak et al., 2004; Pulvers et al., 2010). Furthermore, recent evidence suggests that spindle orientation itself does not affect the fate of the progenitor, but only the inheritance of apical junctions and thus its position relative to the VZ (Konno et al., 2008; Morin et al., 2007). In the spinal cord both symmetric proliferative and asymmetric neurogenic divisions display a wide range of cleavage plane orientations (Wilcock et al., 2007). Thus the actual relevance of spindle positioning in the maintenance of neuronal progenitors remains ambiguous.

1.2.3.7. Lateral inhibition

When neurogenesis begins, proneural transcription factors such as Ngn2 and Mash1 directly upregulate expression of Notch ligands, for example *Dll1* (Castro et al., 2006). These ligands in turn activate Notch signaling in the neighboring cell, which activates Notch target expression such as *Hes* transcription factors (**Figure 2**). Hes proteins regulate their target genes, such as proneural genes, by active and passive repression (Kageyama et al., 2007). In the active repression, Hes factors bind to target gene promoters together with co-repressors of Tle/Grg family, and inactivate chromatin. In the passive repression, Hes factors bind to proneural proteins such as Mash1 and E47, and prevent their binding to their target E-box in DNA. Hes1 can also promote G1 phase by inhibiting CKIs such as p21 and p27 (Kabos et al., 2002; Murata et al., 2005). As a consequence, the cell with active Notch-signaling stays as a proliferative progenitor. These communication events, known as lateral inhibition, result in neighboring cells adopting different fates, as the cell with active Notch signaling stays

as a proliferative progenitor. The lateral inhibition was first described in *Drosophila* and *C. elegans* (Heitzler and Simpson, 1991; Wilkinson et al., 1994; Heitzler et al., 1996), but it operates also in vertebrate CNS (Kawaguchi et al., 2008; Kageyama et al., 2008).

According to the classic lateral inhibition model, neuronal progenitors are initially equivalent and express same level of proneural genes and Notch ligands (**Figure 7A**). However, small stochastical differences result in some cells expressing more of these genes, which results in more efficient Notch activation in the neighboring cells. Subsequent reciprocal interactions then lead to differences in Notch signaling between cells, and the cell which expresses higher amount of ligands will be selected as a neuronal precursor, while the neighboring cells remain as progenitors. Thus, cells which initially express slightly higher amounts of proneural genes become selected as neuronal precursors. Indeed, many components of Notch signaling and their proneural regulators show a "sal t-and-pepper" expression pattern in the developing neuroepithelium (Lindsell et al., 1996; Kageyama et al., 2008; Kawaguchi et al., 2008; Shimojo et al., 2011; see also **Figure 7C**), supporting the lateral inhibition model.

However, recent observations have challenged the traditional view of lateral inhibition (Shimojo et al., 2008; Kageyama et al., 2008; Shimojo et al., 2011). The observed "salt-and-pepper" expression pattern represents only a snap-shot of gene expression, while in reality the expression levels fluctuate (**Figure 7B**). Notch target Hes1 is able to repress its own transcription, but as the protein's half-life is very short, the repression is relieved rather rapidly. As a co nsequence, *Hes1* oscillates in a p eriod of 2-3 hours in neuronal progenitors (Hirata et al., 2002; Shimojo et al., 2008). Only exception to this appears to be progenitors in boundary regions, such as in the midbrain-hindbrain boundary. In these regions, *Hes1* is constantly expressed, which suppresses both cell proliferation and neurogenesis (Baek et al., 2006). This may be accomplished by t he ability of continuously expressed *Hes1* to prevent cell cycle progression by repressing *CyclinD1* and *CyclinE2*. Currently it is not known how oscillation vs. stable expression of *Hes1* in different areas is accomplished. Suggested mechanisms include regulation by Jak-Stat-pathway or HLH-factor *Id* (Shimojo et al., 2011).

In neuronal progenitors, cyclically expressed Hes1 regulates *Ngn2*, which in turn directly regulates *Dll1* (Shimojo et al., 2011). Consequently, the expression of *Ngn2* and *Dll1* also displays oscillation, which has been demonstrated with real-time imaging (Shimojo et al., 2008). However, in postmitotic neurons *Ngn2* and *Dll1* are continuously expressed.

Hes1 is expressed in neuroepithelial cells from E7.5, and appears initially to be independent of Notch signaling (Kageyama et al., 2007). Later *Hes1* expression continues in radial glial cells. Other Notch targets in neuroepithelium include *Hes3, Hes5*, and *Hes6*. *Hes3* is expressed in neuroepithelial cells, while *Hes5* is expressed in radial glia. Basal progenitors in the forebrain do not express *Hes1* or *Hes5* (Mizutani et al., 2007; Kawaguchi et al., 2008), but these genes are expressed in the OSVZ progenitors (Hansen et al., 2010). The presence of Notch signaling might direct subventricular cells into becoming OSVZ progenitors, while the absence of Notch would guide them to adopt basal progenitor fate (Shimojo et al., 2011). Hes-related bHLH factors Hesr1 and Hesr2 (Hey/Herp/CHF) appear also to maintain neuronal progenitors, and are regulated by Notch signaling (Sakamoto et al., 2003; Iso et al., 2001). They can form heterodimers with Hes factors and act as repressors.

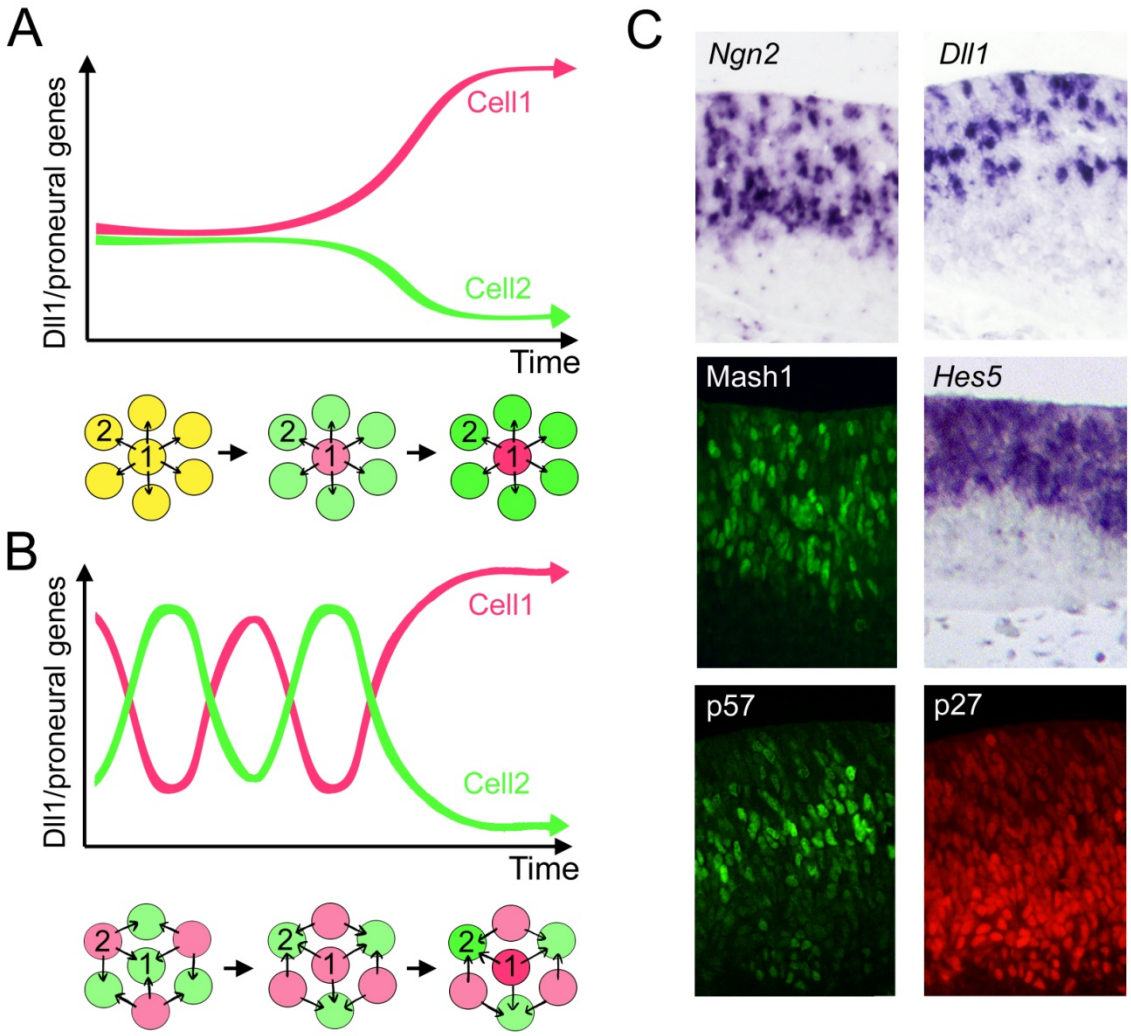


Figure 7. Oscillation of Notch signaling pathway components and lateral inhibition.
(**A**) Traditional view of lateral inhibiton, based on studies in *Drosophila*. In the beginning, all cells contain equal amount of proneural genes, Notch receptors, and Notch ligands. Small stochastical variations result in one cell having more proneural genes (pink). This cell is selected as a neuronal precursor cell which inhibits the differentiation of surrounding cells (green). (**B**) Updated view of lateral inhibiton. *Hes1*, *Dll1*, and *Ngn2* show oscillatory expression in neuronal progenitors. By an unknown mechanism, some cells are induced to exit the cell cycle, which stabilizes the expression of proneural genes and Notch ligands. (**C**) "Salt-and-pepper" expression pattern of Notch-signaling components, proneural genes, and negative cell cycle regulators in E10.5 ventral midbrain VZ. Apical side upwards. (A) and (B) are based on models in Kageyama et al., (2008).

Hes1, 3, and *5* are able to some extent compensate for each other, but inactivation of all three results in premature neurogenesis and early depletion of neuronal progenitor pool (Hatakeyama et al., 2004). In these mutants, proneural genes *Mash1* and *Ngn2* are highly upregulated. In contrast, Hes6 functions in an opposite manner by inhibiting Hes1 function and thus promoting neurogenesis (Bae et al., 2000). Its expression is induced by proneural factors (Koyano-Nakagawa et al., 2000). Later in embryogenesis Hes1 and Hes5 are required for the formation of astrocytes. Thus, the function of Hes factors is likely to ensure that neuronal progenitors are maintained so that all neuronal and glial cell types are produced.
If both Notch-signaling and proneural genes are cyclically expressed, what determines the timepoint of cell-cycle exit? One possibility is that gene expression oscillation functions as a cellular clock, leading to gradual accumulation of factors such as Cend1 (BM88), which induce the cell cycle exit (Politis et al., 2007). Alternatively, cell-cycle-coupled phosphorylation state of proneural proteins may act as a timer to control induction of neurogenesis (Ali et al., 2011).

1.2.3.8. Transcriptional control in self-renewal and neurogenesis

Proneural bHLH genes *Ngn1*, *Ngn2* and *Mash1* are expressed in neuronal progenitors and newly differentiated precursors, but their expression is switched off in mature neurons (Bertrand et al., 2002; Guillemot, 2007). Together with ubiquitous E47 transcription factors, proneural proteins bind to their targets as heterodimers and activate gene expression. Overexpression of proneural genes promotes neuronal differentiation. They also can regulate neuronal subtype specification together with homeodomain factors and other bHLH factors, as well as suppress alternate fates (Kageyama et al., 2005). Additionally, proneural proteins can induce neuronal migration, and even promote cell cycle progression (Castro et al., 2011; Pacary et al., 2011).

Same proneural protein can have opposite functions depending on the cellular context, which is accomplished by different phosphorylation patterns. For example, phosphorylated Olig2 has antineurogenic functions, whereas in non-phosphorylated state Olig2 promotes differentiation (Sun et al., 2011). Phosphorylation of Ngn2 at serine residues S231 and S234 promotes motoneuron specification (Ma et al., 2008), whereas its phosphorylation at tyrosine Y241 promotes neuronal migration (Hand et al., 2005), and at multiple proline-serine residues prevents neurogenesis (Ali et al., 2011).

Proneural genes are expressed in the developing neuroepithelium well before the onset of neurogenesis (Bettenhausen et al., 1995; Hatakeyama and Kageyama, 2006; Hatakeyama et al., 2004; Guillemot and Joyner, 1993). How is it possible, then, that this early expression does not induce neuronal differentiation? One possible explanation is that the cyclical expression of proneural genes, described above, is not long enough to promote neurogenesis, but is only able to induce quickly responding genes, such as *Dll1* (Shimojo et al., 2011). Only when expression of *Ngn2* becomes sustained, it can induce neuronal differentiation. Additionally, the above mentioned phosphorylation state of Ngn2 may regulate target activation (Ali et al., 2011).

Sox family consists of 20 transcription factors, divided into eight subgroups A-H (Bowles et al., 2000). Sox proteins work as dimers with other transcription factors, which may be either Sox members, or factors belonging to other families, such as Oct (Kamachi et al., 2000). The binding partners affect the target specificity and function, as

same Sox protein can have different functions in different cells, or it can regulate different events in the same cell (Kiefer, 2007). Furthermore, the activity of Sox proteins is controlled by posttranslational modifications as well as the presence of activators and inhibitors.

SoxB1 family members Sox1, 2, and 3 antagonize the function of proneural genes in a Notch-independent manner (Bylund et al., 2003; Pevny and Placzek, 2005; Holmberg et al., 2008). In turn, proneural factors can directly bind and inhibit SoxB1 protein function (Guillemot, 2007). *SoxB1* genes are expressed widely in neuronal progenitors both during embryogenesis and in the adult brain, and their overexpression inhibits neurogenesis. Concomitantly, their inactivation leads to premature neuronal differentiation, and depletion of the progenitor pool (Bylund et al., 2003; Graham et al., 2003; Ferri et al., 2004).

In contrast to Sox1-3, the members of SoxB2 group, Sox14 and Sox21, promote neurogenesis. Proneural factors upregulate Sox21, which after reaching a threshold level, is able to inhibit Sox1-3 function (Sandberg et al., 2005). Sox21 might also induce neurogenesis by directly suppressing SoxB1 targets (Kiefer, 2007). Furthermore, SoxC group proteins Sox4 and Sox11 are required for the manifestation of neuronal properties in postmitotic neurons (Bergsland et al., 2006).

The role of other Sox subfamilies, such as SoxD proteins, has been well characterized in the formation of peripheral nervous system. These factors could likely play a role also in the formation of CNS. Indeed, recently it was shown that Sox9 is involved in the induction and maintenance of neuronal progenitors, and Sox5 regulates sequential generation of neuronal subtypes in the cortex (Lai et al., 2008; Scott et al., 2010).

Direct targets of Sox proteins are less well known. One possible mechanism for Sox action is modulation of Wnt-beta-catenin pathway (Kormish et al., 2010). For example, Sox5 is needed for regulation of cell cycle progress in neuronal progenitors, where it interferes with Wnt-signaling (Martinez-Morales et al., 2010).

1.2.3.9. Extracellular signals affecting the balance between self-renewal and differentiation

Several major signaling pathways have been associated in the regulation of proliferation vs differentiation decisions. However, direct evidence on how these signals regulate intracellular effector molecules is scarce.

1.2.3.9.1. Shh

Shh signaling may promote proliferation of neuronal progenitors, as *Gli3* and *Gli2* mutant tissue forms less neurospheres than wild-type tissue (Palma and Ruiz I Altaba, 2004). Hh signaling stimulates proliferation in retina and in cerebellar granule cells (Amato et al., 2004; Locker et al., 2006; Fuccillo et al., 2006; Sakagami et al., 2009), and it maintains neuronal stem cells in the developing hippocampus (Favaro et al., 2009). The effect of Shh extends to adult neuronal stem cells, where it regulates their self-renewal (Ahn and Joyner, 2005). In general, Hh signaling affects the expression of cell cycle machinery components, such as *Cyclins*. Other suggested links between Shh and cell cycle regulation include N-myc, E2F1 and E2F2 (Fuccillo et al., 2006). In retina, Hhs also regulate cell type specification via proneural genes such as *Math5* (Sakagami et al., 2009). The effect of Hh signaling appears to depend on context – in

some tissues, Shh acts a mitogen, whereas in other regions, such as ventral midbrain, it inhibits cell proliferation (Joksimovic et al., 2009b).

1.2.3.9.2. Wnts

Proliferation and differentiation decisions in neuronal progenitors also involve beta-catenin-mediated Wnt-signaling (Doe, 2008). The mitogenic effect may result from a synergistic function with FGF2, although evidence appears controversial. Studies of the developing cortex have identified one regulator of neuronal progenitor self-renewal as *Wnt3a* (Munji et al., 2011). Overexpression of this gene promoted also mitoses in basal progenitors, resulting in increased neuron production. This mitotic effect on neurogenic basal progenitors may partly explain earlier results, which showed that during early embryogenesis (E10), Wnt signaling promotes self-renewal of neuronal progenitors, whereas later (E14) it promotes neuronal differentiation (Chenn and Walsh, 2002; Chenn and Walsh, 2003; Hirabayashi and Gotoh, 2005).

The function of Wnt-beta-catenin pathway has mostly been studied by a conditional stabilization or inactivation of *beta-catenin*. Reducing the level of beta-catenin signaling results in premature neurogenesis and loss of neuronal progenitors (Machon et al., 2003; Zechner et al., 2003). Stabilized expression of *beta-catenin* in mice leads to enlargened brain, due to expansion of progenitor population (Chenn and Walsh, 2002; Chenn and Walsh, 2003). This effect is cell-autonomous (Woodhead et al., 2006; Chilov et al., 2011).

As beta-catenin is also a structural component of the adherens junctions, distinguishing the actual effects of Wnt signaling from secondary defects, caused by the disruption of adherens junctions and loss of polarity, has proved challenging. In addition, beta-catenin appears to have additional functions in progenitors. Phosphorylated beta-catenin localizes in the centrosomes of mitotic neuronal progenitors and helps to regulate spindle orientation (Chilov et al., 2011). In a chimeric mouse VZ, areas with decreased amount of beta-catenin but intact apico-basal polarity displayed more asymmetric cell divisions, resulting in increased neuron production.

1.2.3.9.3. FGFs

Despite the fact that FGFs have been identified as mitogens *in vitro* (Vescovi et al., 1993; Kitchens et al., 1994), their role in controlling the proliferation and differentiation decisions in the CNS has began to receive more attention only recently. One of the main reasons was the early lethality in many *Fgfr* and *Fgf* null mutant mice, which hampered functional analysis of FGF signaling in later embryonic stages. Generation of conditional alleles of *Fgfrs* and several FGF ligands has helped to overcome the early lethal effects of null alleles, and together with overexpression approaches provided a valuable tool for dissecting the role of FGFs during CNS development.

In the neural plate, FGF signaling maintains the early spinal cord “stem zone”, via interacting with Notch signaling (Akai et al., 2005; Delfino-Machin et al., 2005). Similar interaction of FGFs and Notch has been observed in the developing forebrain (Yoon et al., 2004). On the other hand, FGF signaling can regulate Notch target *Hes1* via FRS2alpha-ERK1/2 route, independently of Notch signaling (Sato et al., 2010). Overexpression of FRS2alpha via electroporation induced *Hes1*, stimulated progenitor self-renewal, and prevented differentiation. FGFs might also inhibit neuronal differentiation by accelerating cell cycle progression (Wilcock et al., 2007).

During early development of the forebrain, FGF signaling maintains patterning and survival of progenitors. Loss of FGF signaling in this early stage leads to extensive

apoptosis and loss of forebrain structures (Paek et al., 2009). Later FGFs maintain self-renewal of progenitors (Maric et al., 2007; Kang et al., 2009; Stevens et al., 2010). Conditional inactivation of *Fgfr1-3* in the forebrain radial glia results in premature transformation of these cells into more committed precursors (Kang et al., 2009). Even the inactivation of only *Fgfr1* in these cells results in premature neurogenesis, although the effect is less pronounced compared to compound mutants (Shin et al., 2004; Müller et al., 2008). These mice display a loss of specific neuronal subtypes, which manifests as hyperactivity.

Interestingly, inactivation of *Fgf10* in the forebrain initially delays neurogenesis (Sahara and O'Leary, 2009), but mutant radial glia eventually begins to produce neurons and do it in excessive amounts. Thus, FGF10 likely regulates timing of neurogenesis, but other FGFs are likely needed for the maintenance of progenitors. Other candidates include FGF2, whose loss reduces both proliferation and neurogenesis of cortical progenitors (Vaccarino et al., 1999; Raballo et al., 2000), and FGF8 (Borello et al., 2008). In contrast, FGF15 antagonizes FGF8 and promotes neurogenesis (Borello et al., 2008). Same phenomenon has been observed in the midbrain, where inactivation of *Fgf15* leads to downregulation of proneural genes and a failure to exit cell cycle (Fischer et al., 2011).

Taken together, neuronal progenitors are affected by multiple convergent signaling pathways, which may interact with many intracellular effectors of different signaling cascades. Understanding neuronal progenitor biology requires detailed analysis of the convergence of these signals.

1.3. Patterning of the midbrain-hindbrain region

After the formation of the neural plate, signals from the anterior visceral endoderm (AVE) have provided it with an anterior character. One of these characteristics is the expression of a homeodomain gene *Otx2*, which is initially expressed in the entire epiblast, and by the end of gastrulation in the anterior neural plate (Ang et al., 1994; Simeone et al., 1993; Bally-Cuif et al., 1995; Pannese et al., 1995; Acampora et al., 1995). Without *Otx2*, forebrain and midbrain fail to develop (Acampora et al., 1995). *Otx2* and *Gbx2* repress each other, which results in the formation of expression boundary in the border of midbrain and hindbrain (see below).

Other signals from AVE include Wnt, BMP and Nodal antagonists, for example Cerberus and Dickkopf, which protect forebrain from caudalizing influences, and help to establish a seco ndary signaling center: anterior neural ridge (Perea-Gomez et al., 2001; Levine and Brivanlou 2007).

In order to allow the development of more posterior brain structures, such as midbrain and hindbrain, the anteriorizing signals must be opposed by posteriorizing factors, such as Wnts, retinoic acid and Nodals. These factors originate in more posterior mesendodermal tissue. One such caudalizing signal is *Wnt8*, which represses *Otx2* and induces *Gbx2* expression in the future hindbrain (Rhinn et al., 2005).

Thus, the initial specification of different central nervous system territories begins already at the neural plate stage. After the establishment of initial brain pattern, the

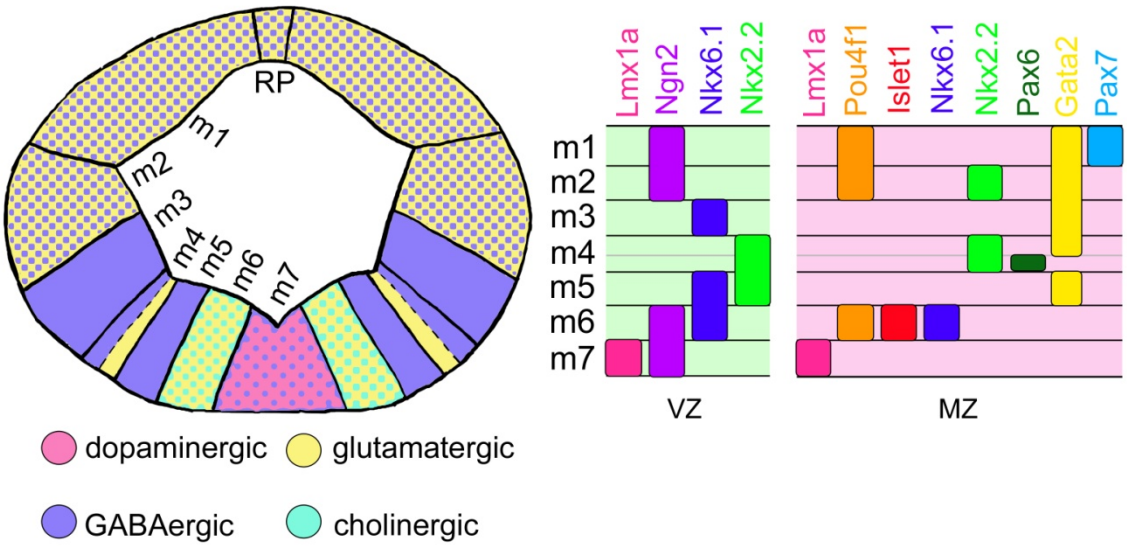


Figure 8. D-V domains in the embryonic midbrain.
Schematic drawing of a coronal section of E12.5 mouse midbrain, showing a division into seven domains m1-m7, originally described in Natatani et al., (2007). Color-code shows the neuronal types produced in each domain, based on the type of neurotransmitter that they release. Some domains produce several types of neurons (dots). Note that m4 is divided into ventral and dorsal halves (dashed line). Each domain is characterized by a combination of transcription factors, here shown only some of them. RP, roof plate. Based on Kala et al., (2009) and a personal communication with K. Achim (Kala).

different parts of the brain continue to develop largely as independent units. Soluble molecules secreted from local signaling centers regulate the patterning and regional identity of forebrain, midbrain and hindbrain.

1.3.1. D-V patterning of the midbrain and hindbrain: the role of floor plate and roof plate

D-V patterning of the neural tube has been most studied in the developing spinal cord, where dorsalizing BMPs from the roof plate counteract the ventralizing Shh from the notochord and floor plate. The gradients of these morphogens then determine the activation of transcription factor code along the D-V axis, resulting in the formation of specific pattern of various motor and interneurons (Ribes and Briscoe, 2009). Roof plate also expresses *Wnt1* and *Wnt3a*, which regulate cell proliferation in the dorsal regions (Megason and McMahon, 2002).

Dorso-ventral patterning of midbrain and hindbrain share several characteristics with the spinal cord, including the use of signals from roof plate and floor plate (Alexandre and Wassef, 2005). In addition, indirect signals from the A-P signaling centers might be involved. For example, evidence from chick and zebrafish suggests that FGF8 from the isthmic organizer participates in the D-V patterning of midbrain, by inducing both roof plate and floor plate formation (Alexandre and Wassef, 2005).

1.3.1.1. Roof plate

During neurulation, roof plate is formed from the lateral edges of the neural plate, which brings together laterally located domains of *Bmp*-expressing non-neuronal ectoderm. The surface-derived BMPs then induce *Bmp* expression also in the underlying dorsal neural tube.

The dorsal midbrain and hindbrain express gradients of several *Bmps* (Solloway and Robertson, 1999; Alexandre et al., 2006). In the spinal cord, the loss of *Bmp* expression leads to the expansion of ventral neural fates and loss of dorsal neurons. However, in the D-V patterning of midbrain and hindbrain these signals remain poorly studied, mainly because of their graded expression and the scarcity of easily identifiable nuclei. In mouse, locus coeruleus in the dorsal rhombomere 1 requires BMP5/7 from the roof plate, together with isthmic signals (Tilleman et al., 2010; Vogel-Höpker and Rohrer, 2002).

3.1.2. Floor plate

In mouse, floor plate develops from the ventral side of the neural tube. During gastrulation, node-derived notochord develops under the midline of neural tube. It secretes glycoprotein Shh, and induces *Shh* expression in the above floor plate, which then functions as a v entral signaling center (Patten and Plackzek, 2000). Shh is best known for its ability to induce ventral structures of the spinal cord, opposing the roof plate -derived BMPs, but it also acts as a mitogen (Jessell, 2000; Rowitch et al., 1999). However, blocking Shh signaling in the chick midbrain increases proliferation and reduces differentiation (Bayly et al., 2007), and in mouse, the loss of *Shh* in the hindbrain increases both proliferation and neurogenesis (Joksimovic et al., 2009b). The latter effect appears to result from upregulation of Wnt-pathway.

In $Shh^{-/-}$ embryos, ventral structures of neural tube fail to form (Chiang et al., 1996), although simultaneous inactivation of the Shh pathway repressor, *Gli3*, is able to rescue majority of the ventral cell types (Litingtung and Chiang, 2000). Similarly, Shh regulates formation and patterning of ventral midbrain structures via target activator *Gli2A* (Agarwala et al., 2001; Fedtsova and Turner, 2001; Blaess et al., 2006). Furthermore, Shh target repressor *Gli3R* is required for the establishment of dorsal midbrain and hindbrain structures, as well as for restricting *Fgf8* domain to the isthmus (Blaess et al., 2006; Blaess et al., 2008). Thus, *Gli3* appears to coordinate the organizer activities of the floor plate and isthmus. In addition, Shh regulates the development of dorsal structures also in the diencephalon and anterior midbrain via *Fgf15* (Ishibashi and McMahon, 2002).

1.3.1.3. Dorso-ventral domains in the midbrain

Similarly to the spinal cord, both chick and mouse midbrain can be divided into dorso-ventral domains (Agarwala and Ragsdale, 2002; Nakatani et al., 2007; Kala et al., 2009; see also **Figure 8**). These domains, also termed midbrain arcs, express specific combinations of transcription factors in the VZ and MZ. These domains are also named m1-m7, with m7 being the most ventral one corresponding to *Lmx1a/b* expression at E12.5 (Nakatani et al., 2007; Kala et al., 2009). Each of these domains is specified by a unique transcription factor code, and gives rise to a specific subset of neurons – m7 produces dopaminergic neurons, whereas the neighboring m6 gives rise to both cholinergic $Islet1^{+}$ motor neurons and glutamatergic $Pou4f1^{+}$ neurons of red nucleus

(Nakatani et al., 2007; Kala et al., 2009). Domains m5-m1 produce GABAergic and glutamatergic neurons. Contribution of these neuronal precursors to specific nuclei is currently poorly understood.

1.3.2. Neuromeric model and the A-P pattern of midbrain and hindbrain

In the antero-posterior direction, the CNS can be divided into repeating metameric units called neuromeres (**Figure 9**). The nowadays widely accepted neuromeric model, which has its origins in the 19th century, was originally based on morphological features of the developing CNS. Addition of gene expression data has lead to the formulation of the current model (Puelles and Rubenstein, 2003).

1.3.2.1. Hindbrain

Neuromeres are especially noticeable in the developing hindbrain, where they are called rhombomeres. Rhombomeres represent lineage-restricted compartments, i.e. cell-mixing between the rhombomeres is inhibited (Fraser et al., 1990). An ordered pattern of *Hox*-genes, together with other transcription factors such as *Krox20*, provide segmential identity in the hindbrain. *Hox*-genes are expressed caudally from rhombomere 2, being absent in rhombomere 1. Isthmic FGF8 might repress the anterior boundary of *Hox* gene expression, thus regulating the size of rhombomere 1 (Irving and Mason, 2000).

1.3.2.2. Diencephalon

Similar segments have been identified in the developing diencephalon, which consists of three prosomeres p1-p3 (Puelles and Rubestein, 2003). According to this model, prosomere 1 is the most caudal one, i.e located next to the midbrain, and it contains pretectum (**Figure 9**). Prosomere 2 refers to the thalamus and epithalamus, and prosomere 3 consists of prethalamus and eminentia thalamus. However, it should be noted that these domain boundaries are a subject to interpretation and other models have been proposed (reviewed in Lim and Golden, 2007). Initial cell-labeling injections by Figdor and Stern (1993) suggested that early prosomeres may represent lineage-restricted domains. However, later studies using retrovirus-labeled clones contradicted those results (Arnold-Aldea and Cepko, 1996; Golden and Cepko, 1996). Lineage-restriction is likely present in proliferating progenitors, but subsequently disappears in postmitotic precursors. Furthermore, similarly to the situation in rhombomeres, some cells are able to escape boundary restrictions (Lim and Golden, 2007).

A local signaling center zona limitans intrathalamica (ZLI) is formed between prosomeres 2 and 3, at the border where prethalamic plate and epichordal plate (future notochord) meet (Echevarría et al., 2003), and from there it extends dorsally. Tissue-transplantion and in ovo electroporation studies have demonstrated that ZLI regulates patterning of diencephalon, and that Shh is the main patterning molecule (Kiecker and Lumsden, 2004; Vieira et al., 2005). Also Wnt and FGF signaling are important for ZLI function (Lim and Golden, 2007). Studies by Lumsden and colleagues identified ZLI as a separate compartment, instead of being a mere border between prosomere 2 and prosomere, and suggested that ZLI is the only lineage-restricted region in the diencephalon (Larsen et al., 2001).

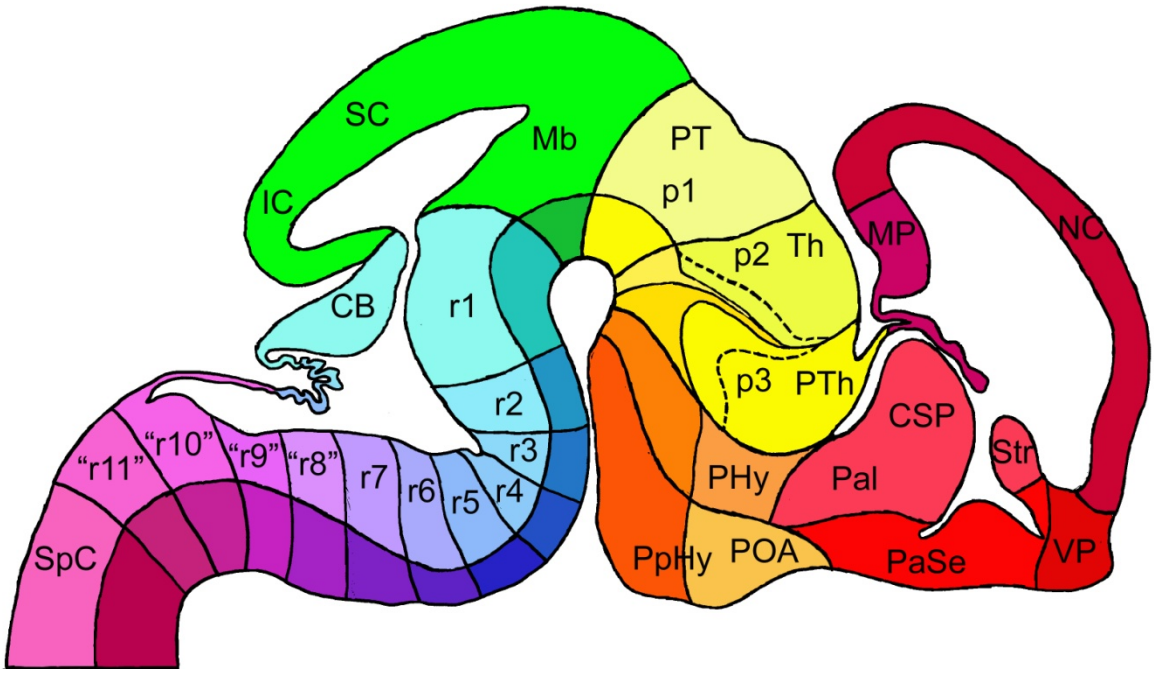


Figure 9. Neuromeric divisions in the embryonic mouse brain.

Sagittal view of E13.5 brain, showing some of the antero-posterior subdivisions. Forebrain (orange and red regions) is the most complex unit, and here are shown only some of the structures. Most anterior forebrain will form the neocortex (NC), and posterior region forms hypothalamus (Hy) which here is divided into two parts. Diencephalon (yellow) is divided into three prosomeres (p1-p3). Dashed line indicates p2ZL and p3ZL, which are subdomains of prosomeres 2 and 3, respectively. Midbrain (green) is one neuromere (mesomere). Dorsal midbrain forms superior and inferior colliculi (SC and IC). Hindbrain is divided into seven rhombomeres (r1-r7) and additional four pseudorhombomeres ("r8"-"r11"). Cerebellum (CB) is derived from rhombomere 1. Basal plate is indicated with a darker colour from hypothalamus to spinal cord (SpC). POA, preoptic area; PT, pretectum; Th, thalamus; PTh, prethalamus; PHY; peduncular hypothalamus; PpHy, prepeduncular hypothalamus; PaSe, paraseptal subpallium; CSP, central subpallium; Pal, pallidum; Str, striatum, VP, ventral pallium, MP, medial pallium (hippocampal allocortex). Neuromeric model by Puelles and Rubenstein (2003), pseudorhombomeres described in Marín et al (2008). Picture re-drawn and modified from E13.5 reference map in The Allen Brain Atlas.

1.3.2.3. Midbrain

In contrast to the hindbrain and diencephalon, midbrain consists of one compartment: mesomere (**Figure 9**). Already at stage 10 in chick, the mesencephalic vesicle (midbrain) can be morphologically distinguished from the prosomeric vesicle (diencephalon) (Garcia-Lopez et al., 2004)**.** In the dorsal midbrain, the posterior commissure – a neuron bundle which traverses the border between tectum and pretectum – defines the morphological boundary between the midbrain and the diencephalon. Genetically, the dorsal boundary between tectum and pretecum is determined by the cross-repression between FGF8-regulated En1 in the midbrain, and Pax6 in the diencephalon (Araki and Nakamura, 1999; Mastick et al., 1997; Matsunaga et al., 2000; Warren and Price, 1997). Ectopic expression of either of these genes in the chick shifts the boundary position. However, similar crossrepression has not yet been demonstrated in the ventral side, where the meso-diencephalic border is less clearly defined – also morphologically.

Caudally, the boundary between midbrain and hindbrain can be morphologically identified by the presence of the isthmic constriction. Initial cell-labeling and re-aggregation experiments in chick suggested that cells were able to cross the midbrain-hindbrain boundary freely (Jungbluth et al., 2001). However, subsequent fate-mapping experiments demonstrated that this was not the case, and that this region is indeed a true lineage-restricted boundary (Zervas et al., 2004; Sunmonu et al., 2011a). In mouse, beta-galactosidase^{+} cells, labeled with an inducible Wnt1-Cre or Gbx2-Cre, do not cross the midbrain-hindbrain border.

Given that some serotonergic neurons, born in the rostral hindbrain, are later found in the caudal midbrain, at least some cell movement is allowed across the boundary in the postmitotic stage. Similar phenomenon has been observed in the hindbrain, where cellular movement is restricted during early development but allowed later (Lumsden, 2004). One candidate to maintain coherence of this boundary is the isthmic FGF signaling. The midbrain-hindbrain boundary contains a narrow population of *Fgfr1*-expressing cells, which also express distinct cell-adhesion molecules (Trokovic et al., 2005). As the loss of these cells leads to visible cell mixing across the boundary, they may be participate in the separation of midbrain and rhombomere 1 cells. Recent results, obtained by using mosaic inactivation of *Fgf8* under an inducible *Gbx2* promoter, support the role of FGF signaling in this process (Sunmonu et al., 2011a).

1.3.3. Regulation of antero-posterior patterning in the midbrain and hindbrain: isthmic organizer

Isthmic organizer, located at the boundary between midbrain and hindbrain, regulates the patterning of midbrain and hindbrain (Lumsden and Krumlauf, 1996; Wurst and Bally-Cuif, 2001; Liu and Joyner, 2001b; Echevarría et al., 2003; Sato et al., 2004). It secretes growth factors which belong to Wnt and FGF families (**Figure 10**).

1.3.3.1. Discovery of the isthmic organizer

The isthmic organizer was discovered in avian explant studies (Alvarado-Mallart et al., 1990; Martinez et al., 1991; Marín and Puelles, 1994; Martinez and Alvarado-Mallart, 1990; Martinez et al., 1995). Isthmic tissue, transplanted into the diencephalon of the host embryo, was able to induce the formation of a second organizer, accompanied with

the isthmus-specific gene expression patterns (Martinez and Alvarado-Mallart, 1990). The tissue anterior to the graft was transformed into ectopic midbrain, whereas caudal tissue turned into hindbrain. When the graft was placed into rhombomeres, it induced the formation of cerebellum (Grapin-Botton et al., 1999). However, in the spinal cord the transplanted isthmic tissue lost its inducing ability, and adopted a more posterior fate.

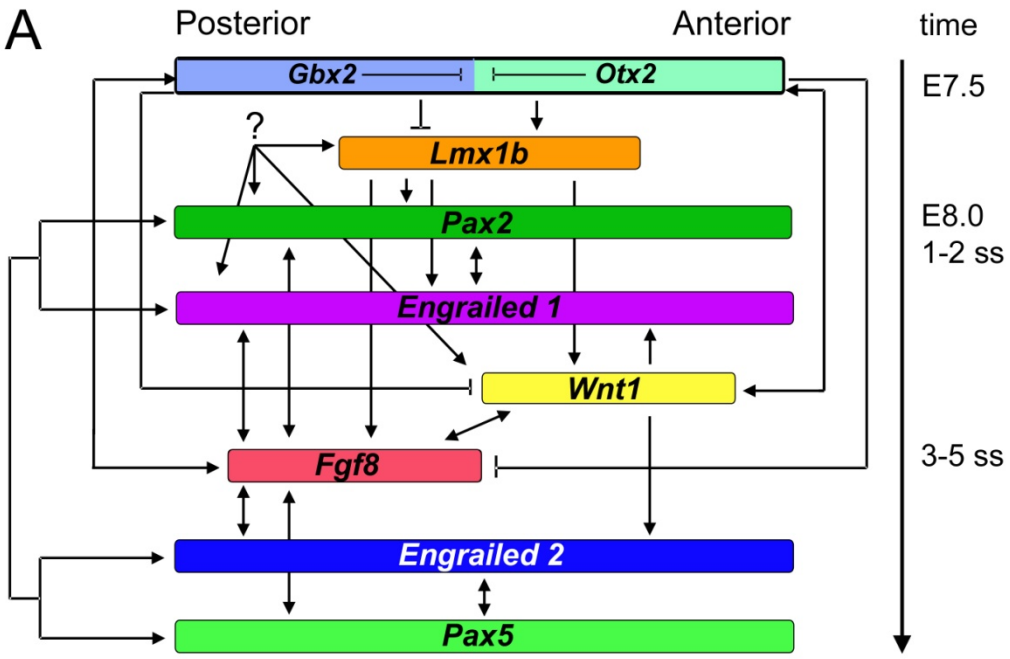
Grafting the transplant anterior to ZLI failed to produce a similar response, suggesting that ZLI forms a competence boundary (Kobayashi et al., 2002). This may be result of *Irx3/Six1* expression: *Irx3*, expressed caudally to ZLI, would give midbrain and prosomeres 1-3 competence to respond to isthmic signals. Similarly, Irx2, whose expression precedes that of *Fgf8* in the midbrain-hindbrain region, prepatterns hindbrain and gives it competence to respond to isthmic FGF8 (Matsumoto et al., 2004). A while later it was discovered that FGF8-soaked beads were able to mimic the patterning activity of the organizer when placed in ectopic locations, which demonstrated that FGF8 is the major signal responsible for isthmic organizer function (Crossley et al., 1996; Irving and Mason, 2000; Martinez et al., 1999; Lee et al., 1997; Liu et al., 1999). Similar results were obtained by the eletroporation of an *Fgf8* construct (Shamim et al., 1999).

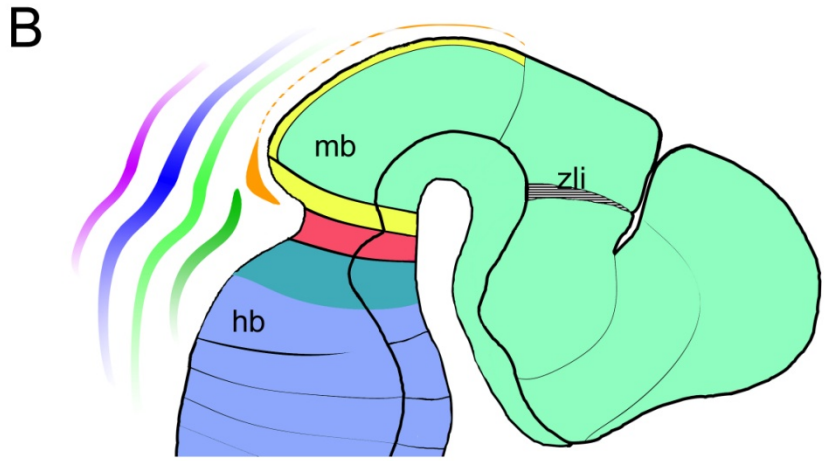
1.3.3.2. Formation of the isthmic organizer

As mentioned above, initial patterning events of the neural plate lead to expression of homeodomain transcription factor *Otx2* in the anterior epiblast, whereas *Gbx2* is expressed in the posterior region. Their mutual repression soon leads to a formation of a sharp boundary at the midbrain-hindbrain region, as *Otx2* is expressed in the prospective midbrain and telencephalon, whereas *Gbx2* is expressed in the hindbrain side (Millet et al., 1999; Broccoli et al., 1999).

At the meeting point of these gene expression domains, the isthmic organizer is formed (Li and Joyner, 2001; Simeone, 2000; Wurst and Bally-Cuif, 2001). Loss of either one of these genes leads to patterning defects in the midbrain-hindbrain region (Acampora et al., 1995; Acampora et al., 1997; Wassarman et al., 1997; Li and Joyner, 2001; Li et al., 2002; Martinez-Barbera et al., 2001). Ectopic expansion of *Otx2* in the hindbrain transforms the rostral hindbrain into midbrain, and shifts the isthmic organizer caudally (Broccoli et al., 1999). Concomitantly, anterior shift of *Gbx2* domain shifts organizer rostrally (Millet et al., 1999).

When *Otx2* and *Gbx2* expressing tissues are juxtaposed, or these genes are ectopically expressed via electroporation, isthmic organizer forms at the new *Otx2/Gbx2* boundary (Irving and Mason, 1999; Bally-Cuif and Wassef, 1994; Hidalgo-Sánchez et al., 1999; Katahira et al., 2000). However, these genes are not required for the initial induction of the organizer, but rather for its correct positioning and maintenance (Millet et al., 1999; Broccoli et al., 1999; Katahira et al., 2000; Li and Joyner, 2001; reviewed in Simeone, 2000). Nevertheless, Gbx2 and Otx2 allow the proper establishment of the isthmic organizer by repressing midbrain- and hindbrain specific genes, respectively (Li and Joyner, 2001). Although the initial signal which induces the isthmic organizer is still unclear, the genetic cascade after the induction has been described in a great detail. The expression of isthmus-specific genes begins at the onset of somitogenesis (**Figure 10A**).


A
Posterior
Anterior
time
Gbx2
Otx2
?
Lmx1b
Pax2
Engrailed 1
Wnt1
Fgf8
Engrailed 2
Pax5
E7.5
E8.0
1-2 ss
3-5 ss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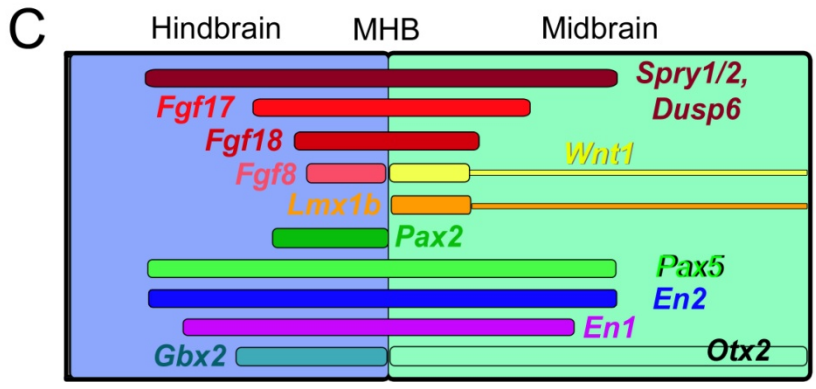
C
Hindbrain
MHB
Midbrain
Spry1/2,
Dusp6
Fgf17
Fgf18
Wnt1
Fgf8
Lmx1b
Pax2
Pax5
En2
En1
Gbx2
Otx2

In mouse, a LIM homeodomain transcription factor *Lmx1b* is expressed in the midbrain-hindbrain region from a very early stage (Guo et al., 2007). Then *Pax2* and *En1* begin to be expressed as broad gradients around the isthmus, and *Wnt1* expression is induced in the caudal part of *Otx2* domain (Joyner et al., 2000). En1 and Lmx1b are likely involved in *Fgf8* induction in the rostral *Gbx2* domain soon after (Reifers et al., 1998; Hidalgo-Sánchez et al., 1999; Mason et al., 2000; Guo et al., 2007). Shortly after, gradients of *En2* and *Pax5* expression are observed around the isthmus (Davis and Joyner, 1988; Davis et al., 1988; Asano and Gruss, 1992; Joyner et al., 2000, Wurst and Bally-Cuif, 2001). In these early stages, expression domains appear blurred and contain large regions of overlap.

After the initiation phase, the isthmic genes form an interdependent genetic network, which maintains the organizer and leads to sharpening of the gene expression domains (Wurst and Bally-Cuif, 2001). The initial broad expression domains become more restricted by E9. *Fgf8* and *Wnt1* are expressed as n arrow bands around the isthmic constriction, and they promote each other's expression (Wurst and Bally-Cuif, 2001; Chilov, 2010; **Figure 10B, C**). The expression of *Lmx1b,* involved in maintenance of the cross-regulatory pathways in the isthmus, becomes restricted to *Wnt1* domain (Adams et al., 2000; Matsunaga et al., 2002; Guo et al., 2007). *En1/2* and *Pax2/5* continue to be expressed as gradients around the isthmus, although also these domains narrow down closer to the isthmus. Together, these genes form a positive feedback loop which maintains a functional isthmic organizer*,* and their loss leads to disruption of the midbrain-hindbrain-region (Wurst et al., 1994; Joyner, 1996; Brand et al., 1996; Favor et al., 1996; Schwarz et al., 1997; Urbanek et al., 1997; Lun and Brand, 1998; Bouchard et al., 2000; Liu and Joyner, 2001a,b; Sato et al., 2004; Wurst and Bally-Cuif, 2001). Concomitantly, ectopic expression of *En1/2* and *Pax2/5* lead to induction of midbrain-hindbrain region genes, including *Fgf8*, followed by f ate-transformation (Araki and Nakamura, 1999; Funahashi et al., 1999; Okafuji et al, 1999; Ristoratore et al., 1999).

Figure 10. Isthmic organizer.

(**A**) Genetic interaction leading to establishment of isthmic organizer. Time indicated on the right. Mutual repression of *Otx2* and *Gbx2* in the neural plate establishes the location of isthmic organizer, but these are not required for its induction. *Lmx1b* is required for the organizer formation, and it induces *Pax* and *En* transcription factors. This activates *Fgf8* in the *Gbx2* domain and *Wnt1* in the *Otx2* region. This interdependent network then is required for organizer function and maintenance. (**B**) Schematic view on E9-E10 mouse brain, showing color-coded expression patterns of isthmic organizer genes. Early broad expression domains have become sharper by mutual inductive and repressive interactions. *En* (blue and purple) and *Pax* (light and dark green) factors are expressed as gradients originating from the isthmus. *Lmx1b* (orange) has become restricted in *Wnt1*-domain (yellow), and is also expressed along the dorsal midline (dashed line). *Fgf8* (red) is expressed in the *Gbx2* expression domain (dark turquoise), next to *Wnt1*. Earlier *Gbx2* expression has defined the entire hindbrain region caudal to isthmus (light blue), whereas *Otx2* is still expressed in the entire midbrain and forebrain (mint green). (**C**) A detailed view of gene expression patterns in (B). Here also other *Fgfs* are shown. FGF17 and FGF18 lack patterning activity. Negative modulators of FGF signaling, such as Sproutys (Spry) and Dusp6 control the spreading of the positive feedback loops. E, embryonic day; ss, somite stage; hb, hindbrain; mb, midbrain; zli, zona limitans intrathalamica, MHB; midbrain-hindbrain boundary. Based on Joyner et al., (2000), Simeone et al., (2000), Wurst and Bally-Cuif (2001); Liu et al., (2003); Guo et al., (2007); Elkouby and Frank (2010).

This genetic network in the isthmus also needs antagonists, which control the extent of the positive feedback loop. Main controllers appear to be the FGF8-induced inhibitors of FGF signaling, such as Sproutys and Dusp6 (Wurst and Bally-Cuif, 2001; Liu et al., 2003; Echevarría et al., 2005). Furthermore, *Grg4* is expressed in chick in a pattern complementary to *En2*, and overexpression studies suggest that it antagonizes isthmic organizer function (Sugiyama et al., 2000).

1.3.3.3. FGF signaling in the midbrain and hindbrain patterning

Partial loss-of-function studies and *in vitro* bead experiments have demonstrated that FGF8 is the main patterning signal of the isthmic organizer (Meyers et al., 1998; Brand et al., 1996; Reifers et al., 1998; Chi et al., 2003). Zebrafish *Fgf8* mutant, *acerebellar (ace)*, which has a point mutation producing a truncated form of FGF8 protein, lacks midbrain-hindbrain boundary and cerebellum (Brand et al., 1996; Reifers et al.,1998). In these mutants, midbrain is expanded caudally and displays abnormal patterning (Picker et al., 1999). In contrast, *Wnt1* secreted from the isthmic organizer appears not to regulate patterning (Adams et al., 2000). Although *Wnt1* mutants lack a large region of midbrain and hindbrain, which leads to death at birth, these defects likely result from the loss of *Fgf8* (McMahon and Bradley, 1990; Thomas and Capecchi, 1990; Mastick et al., 1996; Lee et al., 1997).

Fgf8 null mice die during gastrulation (Meyers et al., 1998; Sun et al., 1999), but hypomorphic *Fgf8* mouse mutants display, among various developmental problems, defects in midbrain-hindbrain boundary formation, and they lack most of midbrain and cerebellum (Meyers et al., 1998). This phenotype greatly resembles that of *ace* mutants. Embryos in which *Fgf8* is conditionally inactivated in the isthmic organizer, show a more severe phenotype where most of the dorsal midbrain and hindbrain region is lost due to extensive apoptosis in an early stage (Chi et al, 2003).,

Of the eight isoforms of *Fgf8* (Crossley and Martin 1995; MacArthur et al., 1995), only *Fgf8a* and *Fgf8b* are expressed in the isthmic organizer (Shamim et al., 1999; Sato et al., 2001). Gain-and loss of function studies have demonstrated that these two splice isoforms possess very different activities (Lee et al., 1997; Liu et al., 1999; Shamim et al., 1999; Sato et al., 2001; Liu et al., 2003; Guo and Li, 2007; Guo et al., 2010; Sunmonu et al., 2011b). In mouse, expression of *Fgf8b* under *Wnt1*-promoter in the prospective midbrain transforms midbrain and caudal forebrain into hindbrain (Liu et al., 1999). In contrast, similar overexpression of *Fgf8a* results only in overproliferation, and upregulation of *En2* (Lee et al., 1997). In chick, misexpressed *Fgf8b* via electroporation prevented midbrain and promoted cerebellum formation in high concentrations, whereas eletroporated *Fgf8a*, *Fgf17*, and *Fgf18* only enlargened the midbrain size (Liu et al., 2003; Sato et al., 2001). Concomitantly, the same overexpression experiments demonstrated that only *Fgf8b* was able to upregulate FGF target genes, such as *Sproutys*, as well as to repress *Otx2* and induce *Gbx2*. Recent loss-of-function experiments in mouse have further demonstrated that *Fgf8b*, but not *Fgf8a*, is essential for the isthmic organizer function (Guo et al., 2010).

Remarkably, these different functions of the two isoforms stem from a change in one amino acid, which changes binding affinity of FGF8 to FGF receptors (Olsen et al., 2006). In addition, these isoforms appear to activate ERK1/2 differently. In chick, overexpressed FGF8a was able to activate ERK only in diencephalon, whereas FGF8b activated it throughout midbrain-hindbrain region and diencephalon (Sato and Nakamura, 2004). This might result from the expression pattern of different FGFRs:

FGFR3, which has a higher affinity to FGF8 than FGFR1, is expressed in the diencephalon and anterior midbrain (Liu et al., 2003; Trokovic et al., 2005; Blak et al., 2005).

Two other FGFs, *Fgf17* and *Fgf18*, are also expressed around midbrain-hindbrain boundary in a domain broader than that of *Fgf8*, and their expression continues after *Fgf8* is downregulated (Maruoka et al., 1998; Xu et al., 2000). However, similarly to *Fgf8a,* these genes regulate proliferation rather than patterning (Xu et al., 2000; Liu et al., 2003; Olsen et al., 2006). $Fgf17^{-/-}$ mice only display a mild cerebellar defect (Xu et al., 2000). Taken together, FGF8b is responsible for the patterning function of the isthmic organizer.

An FGF gradient induces a similarly graded expression of FGF target genes around the midbrain-hindbrain boundary. These include positive and negative modulators of FGF signaling, such as *Dusp6*, *Sproutys* and *Flrts,* and nuclear activators of transcription, such as *Pea3* and *Erm*. However, the distribution of FGF8 protein, and its exact localization in the midbrain VZ, remain incompletely understood. The diffusion mechanism of FGF8 has only recently begun to be elucidated, but has this far only been analyzed in zebrafish and chick (Yu et al., 2009; Chen et al., 2009; Nowak et al., 2011). Detailed studies in zebrafish have revealed that receptor-mediated endocytosis provides a source-sink-mechanism which regulates the distribution of FGF8 (Yu et al., 2009; Nowak et al., 2011). Furthermore, treating chick embryos with alkaline phosphatase - tagged FGFR-antibody revealed a presence of FGF-gradient in the midbrain (Chen et al., 2009).

Of the four *Fgfrs* in the mammalian genome, three (*Fgfr1-3*) are expressed in the developing midbrain and hindbrain at E9-E10 (Walshe and Mason, 2000; Liu et al., 2003; Blak et al., 2005; Trokovic et al., 2005). Whereas *Fgfr1* is expressed throughout this area, *Fgfr2* and *Fgfr3* appear to be more weakly expressed, or entirely absent, in the boundary region. Both can be detected in the anterior midbrain and caudal rhombomere 1. At E9.5, *Fgfr2* is expressed in the ventral floor plate close to the boundary, and its expression extends to ventral midbrain by E12.5 (Blak et al., 2005). *Fgfr3* is absent in the boundary region, but its expression similarly extends to cover entire midbrain by E12.5. Expression of receptors appears to be restricted to the VZ (Trokovic et al., 2005; Blak et al., 2005).

Fgfr1 and *Fgfr2* null mutants die early during embryogenesis due to gastrulation defects (Deng et al., 1994; Yamaguchi et al., 1994; Arman et al., 1998), whereas $Fgfr3^{null}$ mice are viable (Deng et al., 1996). Although no s pecific brain phenotype was found in midbrain-specific $Fgfr2^{cko};Fgfr3^{null}$ mutants, recent analyses have revealed that cortex and hippocampus in $Fgfr3^{null}$ mutant mice are slightly smaller (Blak et al., 2007; Moldrich et al., 2011).

Although same En1-Cre was used to inactivate both alleles, the brain morphology in $Fgfr1^{cko}$ mice is affected less than in $Fgf8^{cko}$ embryos (Trokovic et al., 2003; Chi et al., 2003; Trokovic et al., 2005). $Fgfr1^{cko}$ mice are viable but have problems in motor coordination, likely due to the lack of inferior colliculi and a part of cerebellum called vermis. These findings, together with observed *Fgfr* expression patterns, suggest redundancy between FGFRs in the area.

FGF signaling from the isthmus appears to determine the identity of surrounding cell populations in a dose-dependent manner (Basson et al., 2008; Sato and Joyner, 2009). Misexpression of *Sprouty2* results in cell death in anterior midbrain, but the surviving cells adopt correct positional identity (Basson et al., 2008). Reducing FGF signaling

gradually, via overexpression *Sprouty2* and reducing *Fgf8* dose, leads to an increasing loss of cerebellar vermis tissue. Furthermore, inactivation of isthmic *Fgf8* at different timepoints affects the severity of midbrain-hindbrain phenotype (Sato and Joyner, 2009). Thus, it appears that not only concentration, but also duration, of the isthmic FGF signaling is crucial, and that structures which develop close to isthmus require a longer duration of growth factor signaling.

1.4. Development of the main structures in the midbrain and anterior hindbrain

1.4.1. Cerebellum and locus coeruleus

In adult vertebrates, midbrain and hindbrain form structures of brainstem and cerebellum (**Figure 3**). Cerebellum is derived from rhombomere 1 (Wingate and Hatten, 1999). Glutamatergic external granule cells are born in the rhombic lip and then migrate inwards to form internal granule cells. Inhibitory GABAergic neurons of the cerebellum, such as Purkinje, basket, and stellate cells, originate in the VZ (Hoshino et al., 2005). In addition, cerebellar VZ also produces excitatory glutamatergic neurons of the deep cerebellar nuclei.

The main functions of the cerebellum in the coordination and fine-tuning of movements and controlling balance are well appreciated. Recently it has become clear that cerebellum is involved in the regulation of various other functions, ranging from motor learning to speech and spatial memory (Hatten and Roussel, 2011). As summarized above, signals from the isthmic organizer, especially FGF8, are essential for the formation of cerebellum. In addition, Shh, Notch, and BMPs are involved. Transcription factors regulating neurogenesis in the cerebellum include Math1, Pft1a and Pax2, which have distinct functions in controlling the development of glutamatergic and GABAergic neurons (Carletti and Rossi, 2007).

Another derivative of dorsal rhombomere 1 is locus coeruleus, a nucleus which is the main source of noradrenaline in the mammalian brain. Locus coeruleus projects widely into different parts of the brain and spinal cord. Thus, this nucleus in involved in the regulation of a wide variety of functions, such as anxiety, alertness, sleep/wake cycle, stress, cognition, memory and attention (Singewald and Philippu, 1998; Berridge and Waterhouse, 2003). Malfunction of locus coeruleus has been associated with several psychiatric disorders, such as depression, anxiety and Parkinson's disease (Berridge and Waterhouse, 2003). Although locus coeruleus is born in the dorsal part of rhombomere 1 (**Figure 11**), the progenitor cells migrate ventrally and end up close to the fourth ventricle (Lin et al., 2001; Aroca et al., 2006). BMPs from the roof plate induce *Mash1*, which in turn regulates *Phox2a*, *Phox2b*, and *Ear2*, which are needed for the development of locus coeruleus (Warnecke et al., 2005; Hirsch et al., 1998; Morin et al., 1997; Pattyn et al., 2000).

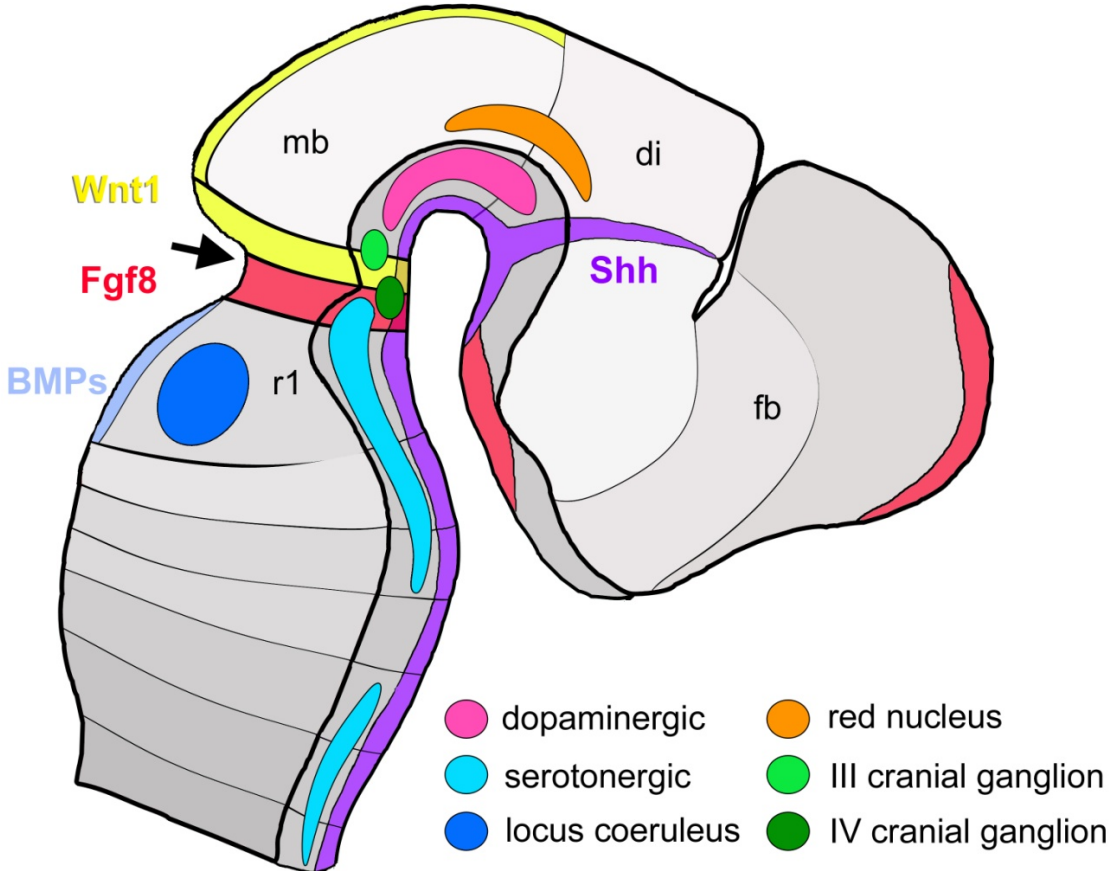

Figure 11. Neuronal nuclei in the embryonic mouse midbrain and hindbrain.
Schematic view on E11 mouse brain with various neuronal nuclei color-coded. Shh from the floor plate (purple), Wnt1 from the isthmic organizer and roof plate (yellow), FGF8 from the isthmic organizer (red), and BMPs from the roof plate (light blue) regulate various aspects in the development of these neuronal populations. The arrow indicates the midbrain-hindbrain boundary. Red nucleus (orange) develops in the basal plate but is here shown above the dopaminergic domain for clarity. Serotonergic neurons in the hindbrain consist of rostral and caudal populations. No serotonergic neurons develop in rhombomere 4. r1, rhombomere 1; mb, midbrain; di, diencephalon; fb, forebrain.

1.4.2. Serotonergic neurons

Serotonergic system is involved in the regulation of mood and behavior. In the adult brain, major serotonergic nuclei are located in the ventral pons and medulla (Rubenstein, 1998). Malfunction of these neurons, or especially defects in serotonin secretion, have been associated with psychological disorders such as clinical depression. Several antidepressants function by blocking the activity of serotonin uptakers, and thus increase the concentration of available serotonin in the synaptic cleft.

Serotonergic neurons are born in the ventral hindbrain between E9.5-E11.5 in mouse (**Figure 11**), and can be identified by expression of serotonin, 5-hydroxytryptamine (5-HT), from E11.5 onwards (Cordes, 2005; Alenina et al., 2006). They are organized into rostral and caudal raphe nuclei, named B9-B1, from anterior to posterior. Rostral raphe nuclei, B4-B9, project into midbrain and forebrain whereas the caudal raphe nuclei B1-3 project towards the spinal cord (Cordes, 2005). The most rostral nuclei, B7-B9, move into the caudal midbrain during late embryogenesis. These nuclei, which develop in the vicinity of the isthmic organizer, are first to be specified already between E9.5-E10.5 in mouse.

Shh and FGF signaling regulate the induction of rostral serotonergic progenitors (Ye et al., 1998). Retinoic acid may also be involved, but this option has been poorly characterized, mainly due to lethality of mouse mutants (Cordes, 2005). The position of midbrain-hindbrain boundary affects the size of serotonergic population (Brodski et al., 2003).

Several transcription factors have been identified which regulate serotonergic neuron development. These include *Mash1*, *Nkx2.2*, *Nkx6.1, Gata2/3*, *Lmx1b* and *Pet1* (Briscoe et al., 1999; Craven et al., 2004; Ding et al., 2003; Pattyn et al., 2004; van Doorninck et al., 1999; Cheng et al., 2003; Hendricks et al., 2003; Kala et al., 2009). Of these, only *Mash1*, *Lmx1b* and likely *Gata2* appear to be indispensable for all serotonergic neurons (Alenina et al., 2006). Especially *Lmx1b* has attracted attention. Forced *Lmx1b* expression in mouse ES cells turns them into serotonergic neurons, and this transcription factor is also needed for serotonin production in the adult brain (Dolmazon et al., 2011; Song et al., 2011).

1.4.3. Superior and inferior colliculi

Dorsal midbrain in mammals forms superior and inferior colliculi (**Figures 3** and **9**). Superior colliculus (optic tectum in birds) is a multilayered sensorimotor structure, which receives most of its input from retina (Agarwala and Ragsdale, 2009). Inferior colliculus (mesencephalicus lateralis dorsalis in chick; torus semicircularis in other vertebrates) processes auditory, and in fish, lateral line sensory information. The sensory information in these regions is organized in ordered structures called topographic maps, and further relayed into higher processing areas in the cortex, or towards the spinal cord. Signals from the roof plate and isthmic organizer set up the gradients of axon guidance molecules such as *Ephrins*, *RGM*, *Wnts,* and their receptors. These molecules then regulate map formation. For example, FGF8-induced En2 regulates *Ephrin* expression (Logan et al., 1996).

1.4.4. III and IV cranial ganglia

Oculomotor complex, which consists of oculomotor nucleus and Edinger-Westphal nucleus, forms the III cranial nerve which controls eye movements. The oculomotor complex originates in m6, a domain which first produces these motoneurons and after that begins to produce neurons of prospective red nucleus (Prakash et al., 2009; see **Figure 8**). Neurons forming the IV cranial ganglion, the trochlear nucleus, develop within the isthmic organizer, and also immediately caudal to it in rhombomere 1 side (Irving et al., 2002; **Figure 11**). This nerve exits brainstem dorsally and projects towards the eye where it innervates the superior oblique muscle. Both III and IV cranial ganglia require Phox2a transcription factor for their formation (Pattyn et al., 1997).

1.4.5. Red nucleus

Red nucleus, which is a part of the cerebellar motor system in the adult brain, contains both glutamatergic and GABAergic neurons. It both sends and receives input from cerebellum and is thus important especially in the control of limb movements (Paxinos, 2004). It consists of two parts: parvocellular domain which is located rostrally and likely originates in diencephalon (Puelles, 1995), and a magnocellular domain which is located more caudally, resides bilaterally in the ventral midbrain, and originates in m6 (also called midbrain arc 1 in chick) (Paxinos, 2004; Agarwala and Ragsdale, 2002; see **Figures 8** and **11**). These two parts are thought to serve a different function, but distinguishing them from each other is difficult in many mammals, because apart from primates, parvocellular part is less clearly defined. Also their exact projection patterns are a topic of controversy. It appears that caudal part gives rise to the rubrospinal tract, whereas more rostrally located cells project to the spinal cord and to the inferior olive. These cells receive input from various regions of the cerebellum but also from other brain areas, such as the sensorimotor cortex and the posterior thalamic nucleus.

Shh from the floor plate and FGF8 from the isthmus regulate the size and positioning of the red nucleus (Fedtstova and Turner, 2001; Agarwala and Ragsdale, 2002). Misexpression of *Fgf8* in chick midbrain shifted both red nucleus and adjacent oculomotoric complex rostrally.

Studies of red nucleus development and characterization in mouse have focused on the midbrain domain – the presumed prospective magnocellular part - and very little is known about the presumed diencephalic domain. Midbrain red nucleus can be identified by the expression of transcription factors *Pou4f1 (Brn3a)* and *Emx2* (Agarwala and Ragsdale, 2002), the latter of which is required for red nucleus development. The midbrain red nucleus progenitors and postmitotic precursors also express *Nkx6.1*, and the loss of this transcription factor leads to abnormal development of this nucleus, although it is not completely abolished (Prakash et al., 2009).

1.4.6. Dopaminergic neurons

Dopaminergic neurons are identified by their production of tyrosine hydroxylase (TH), a rate-limiting enzyme in the dopamine synthesis pathway. Midbrain dopaminergic neurons have been historically classified into three main populations, named A8-A10, which correspond to retrorubral field (RRF), substantia nigra pars compacta (SNpc) and ventral tegmental area (VTA) (Björklund and Lindvall, 1984; Dahlström and Fuxe, 1964; Björklund and Dunnett, 2007; Van den Heuvel and Pasterkamp, 2008). The VTA is located in the most ventral part of midbrain. SNpc is located more laterally and anterior to VTA. In addition, substantia nigra pars reticulata (SNpr) also contains dopaminergic neurons in more caudal regions, but mostly GABAergic neurons in the anterior part (Paxinos, 2004). Retrorubral field is located caudally and dorsally related to substantia nigra.

As dopaminergic neurons are found also in the diencephalon, it has been speculated whether they also originate there (Puelles and Verney, 1998; Verney et al., 2001; Smits et al., 2006, Marín et al., 2005). These midbrain and diencephalic populations are also collectively referred to as meso-diencephalic dopaminergic neurons (reviewed in Smits et al., 2006). Although *Th*-expressing cells can be detected in the caudal diencephalon already at E10.5 (Marín et al., 2005), a detailed fate-mapping of the origins of meso-diencephalic dopaminergic neurons is still lacking.

1.4.6.1. Projections of dopaminergic neurons

Axons from nuclei A8-A10 project to forebrain, forming mesostriatal, mesocortical, and mesolimbic pathways (reviewed in Van den Heuvel and Pasterkamp, 2008; Björklund and Dunnett, 2007). SNpc neurons mainly project to dorsal striatum via nigrostriatal or mesostriatal pathway, and regulate voluntary movements and coordinate motor learning. VTA and RRF neurons project to ventromedial striatum and prefrontal cortex, forming mesocorticolimbic system. These neurons are involved in the control of reward and mood. Moreover, dopaminergic innervations from VTA neurons are required for cell proliferation in adult neurogenic SVZ (Lennington et al., 2011). Midbrain dopaminergic neurons receive input from various brain regions.

The formation of midbrain dopaminergic neuron projections has been well characterized in rat. First neurons are born at E12 (E11 in mouse) and soon after becoming post-mitotic, they start forming projections towards the forebrain. As they exit from the midbrain, dopaminergic neurons form two large axon bundles, called medial forebrain bundles (Van den Heuvel and Pasterkamp, 2008). These bundles also contain non-dopaminergic neurons and travel into the ventrolateral part of the telencephalon. By E14 in rat, dopaminergic neurons reach a region located ventrolateral to the ganglionic eminence/caudate putamen, and a few days later begin to innervate the striatum. Development of dopaminergic axonal projections continues postnatally, when axonal connections are extensively pruned and fine-tuned. In fact, axons originating in VTA and SNpc do not display preference in their target selection between dorsal and ventral striatum, but the topographical specificity is achieved via selective elimination of axons projecting into wrong regions (Hu et al., 2004).

1.4.6.2. Dopamine in neurological disorders

Midbrain dopaminergic neurons are essential for the regulation of movements and cognitive functions. Furthermore, dopamine-containing neuronal circuits regulate concentration, motivation and the ability to experience pleasure. The malfunction or degeneration of these neurons has been associated with several neurogenerative and psychiatric disorders, such as drug addiction, schizophrenia, depression, and Parkinson's disease (Van den Heuvel and Pasterkamp, 2008).

Decreased dopaminergic neurotransmission has been associated with major depression (Dunlop and Nemeroff, 2007). This may be due to diminished dopamine receptor number or function; altered intracellular signaling; or decreased dopamine release from the presynaptic terminals. Midbrain dopaminergic neurons are directly innervated by the serotonergic neurons, and antidepressants which affect the serotonergic system may have an indirect effect on dopaminergic transmission. There is an elevated frequency of depression among patients with Parkinson's disease, which similarly may be directly due to the decreased dopamine levels (Tandberg et al., 1996).

Polymorphisms in genes regulating dopamine synthesis and transmission have been associated with attention-deficit/hyperactivity disorder (AD/HD) (Arime et al., 2011). Dopamine transporter (DAT) mutant mice have been used as an animal model of this disease. DAT is responsible for the clearance of released extracellular dopamine, and thus in $DAT^{-/-}$ mice the level of extracellular dopamine in nucleus accubens and striatum is 10 times higher than normal (Shen et al., 2004). In addition, manganese-enhanced magnetic resonance imaging has revealed that they have alterations in mesocortical

circuitry (Zhang et al., 2010). These mice have impaired memory and learning ability, and display hyperlocomotion in a novel environment (Arime et al., 2011).

Schizophrenic patients display dopaminergic hyperactivity, but also reduced dopamine transmission in the prefrontal cortex (Harrison, 1999; Wong et al., 2003). According to "schizophrenia dopaminergic hypothesis", the increased dopaminergic transmission causes the psychotic symptoms (Davis et al., 1991). Several schizophrenic patients display decreased SNpc and VTA size and cell density (Bogerts et al., 1983). How these brain alterations are linked to the observed increase in dopamine transmission is unknown. It has been suggested that decreased prefrontal activity might cause the dopaminergic hypertransmission, and that the primary cause might be in the abnormal function of basal ganglia (Meyer-Lindenberg et al., 2002; Swerdlow and Geyer, 1998).

Catechol-O-methyltransferase (Comt), which degrades extracellular dopamine, and proline dehydrogenase (Prodh), which catabolises neurotransmitter L-proline, modulate cortical dopamine homeostasis in the frontal cortex (Paterlini et al., 2005). Mice which lack the activity of these enzymes develop schizophrenia-related phenotypes. The genes encoding these two genes are closely located in the chromosomal region 22q11.2, and microdeletions in in this region significantly elevate the risk of schizophrenia (Karayiorgou et al., 1995).

1.4.6.2.1. Parkinson's disease

After Alzheimer's disease, Parkinson's is the most common neurogenerative disease. In this disease, over 60% of dopaminergic neurons in SNpc degenerate and die, and this consequently reduces dopaminergic transmission in striatum by 90% (Lotharius and Brundin, 2002a). Symptoms include tremor at rest, bradykinesia, flexed posture and freezing of gait (Sulzer, 2007). The reason for the onset of Parkinson's disease is unclear, although several hypotheses have been proposed. They range from genetic predisposition to mitochondrial malfunction, and it might be that several mechanisms contribute to the disease (Sulzer, 2007).

At the molecular level, the diseased neurons are characterized by an increased level of alpha-synuclein. This molecule is ubiquitously expressed in neurons, and the protein is located in presynaptic terminals. It is normally required for the synaptic transmission (Lotharius and Brundin, 2002a,b). In the neurons affected in Parkinson's, the improper clearance of alpha-synuclein leads to its abnormal aggregation, and formation of structures called Lewy bodies inside the nerve cells (Spillantini et al., 1997; Lotharius and Brundin, 2002b). How these alterations lead to the degeneration and death of dopaminergic neurons is unclear. It has been suggested that alpha-synuclein might regulate recycling of synaptic vesicles. Aberrant forms of alpha-synuclein would impair the recycling which in turn would lead to the accumulation of cytoplasmic dopamine, which is toxic to neurons (Lotharius and Brundin, 2002b). The Lewy bodies themselves might be a cell's attempt to clear away the mutant protein.

Current treatments of Parkinson's disease focus on alleaviating the symptoms by restoring the cerebral dopamine levels using drugs such as L-DOPA. Replacing the degenerating SNpc neurons in Parkinson's patients has been under intense study, especially by transplanting healthy neurons or neuronal progenitors into patient's brain (Björklund and Dunnett, 2007). However, the transplanted neurons are often unable to efficiently integrate into brain's existing neuronal network and form functional connections. Recently, Studer and colleagues described a more efficient method to engraft dopaminergic neurons, derived from human pluripotent stem cells, into the rodent brain (Kriks et al., 2011). As an alternative to engrafting experiments, one could

attempt to rescue patient's own existing dopaminergic neurons, for example by using neurotropic factors (Diógenes and Outeiro, 2010), reducing neuroinflammation (Tansey and Goldberg, 2010) or inhibiting the function of apoptotic activators (Esposito and Cuzzocrea, 2010). In an even more challenging scenario, neuronal stem cells in the brain could be stimulated to proliferate and differentiate into dopaminergic neurons (Jandial et al., 2009).

All in all, these various approaches may have given promising results in initial animal model studies, but their application in humans has proven to be very difficult. To improve the potential of these possible therapies, the networks of extracellular signals and intracellular effectors controlling the dopaminergic neuron fate must be elucidated in detail.

1.4.6.3. Development of midbrain dopaminergic neurons

Genetic fatemapping has revealed that dopaminergic neurons originate from *Shh*-expressing cells in the ventral midbrain midline (Joksimovic et al., 2009a, Blaess et al., 2011; **Figure 12**). The dopaminergic domain, m7, is further divided into lateral regions and the most medial floor plate, both of which generate neurons (Ono et al., 2007; Bonilla et al., 2008; Joksimovic et al., 2009a; Blaess et al., 2011). As in Parkinson's disease a subset of dopaminergic neurons – in SNpc – is especially vulnerable, midbrain dopaminergic populations may be more heterogenous than initially thought. Indeed, mature midbrain dopaminergic nuclei differ in their gene expression profiles. Neurons in A10 express intracellular Ca^{2+} binding protein, *calbindin1* (Yamada et al., 1990; Parent et al., 1996; Liang et al., 1996), and neuropeptides *Grp*, *Cgrp*, and *Pacap* (Chung et al., 2005b). Genes enriched specifically in the A9 population include *Kcnj6 (Girk2)*, which codes for a G-protein-gated K^+ channel (Schein et al., 1998; Karschin et al., 1996); growth factor *Igf-1*; and mitochondrial protein *Ant-2* (Chung et al., 2005b).

Differences between different precursor domains appear to exist already at very early stages of development. Fate-mapping with an inducible form of Shh-Cre has shown that dopaminergic progenitors in different populations are generated at different timepoints (Joksimovic et al., 2009a; Blaess et al., 2011). In addition, alleged SNpc precursors express transcription factor *Pitx3* before becoming TH^+, whereas VTA precursors behave in an opposite manner (Maxwell et al., 2005).

Several transcription factors participating in dopaminergic neuron specification have been characterized, but their exact role and connection with extrinsinc signals are not well understood (reviewed in Ang, 2006; Prakash and Wurst, 2006a,b; Smidt and Burbach, 2007). These signals and transcription factors likely work in a combination, forming a network regulating different aspects of dopaminergic neuron development, and repressing alternate neuronal fates. For example, Wnt1-Lmx1a are shown to synergistically regulate dopaminergic neuron differentiaton together with Shh-Foxa2 pathway (Chung et al., 2009; Nakatani et al., 2010; Lin et al., 2009; see also **Figure 13**).

Same transcription factor is often present in proliferating progenitors and postmitotic precursors, but it may perform temporally different functions. A transcription factor present widely in progenitors, for example FoxA2, may regulate the initial specification and patterning of the dopaminergic domain, and later regulate survival or the acquisition of the correct neurotransmitter identity.

The development of dopaminergic neurons can be divided into several steps: early regional specification, which is regulated by s ecreted signals from surrounding organizers; adoption of early dopaminergic progenitor fate; and becoming postmitotic and undergoing terminal differentiation (**Figure 12**). In the following, the essential factors affecting these different phases are described.

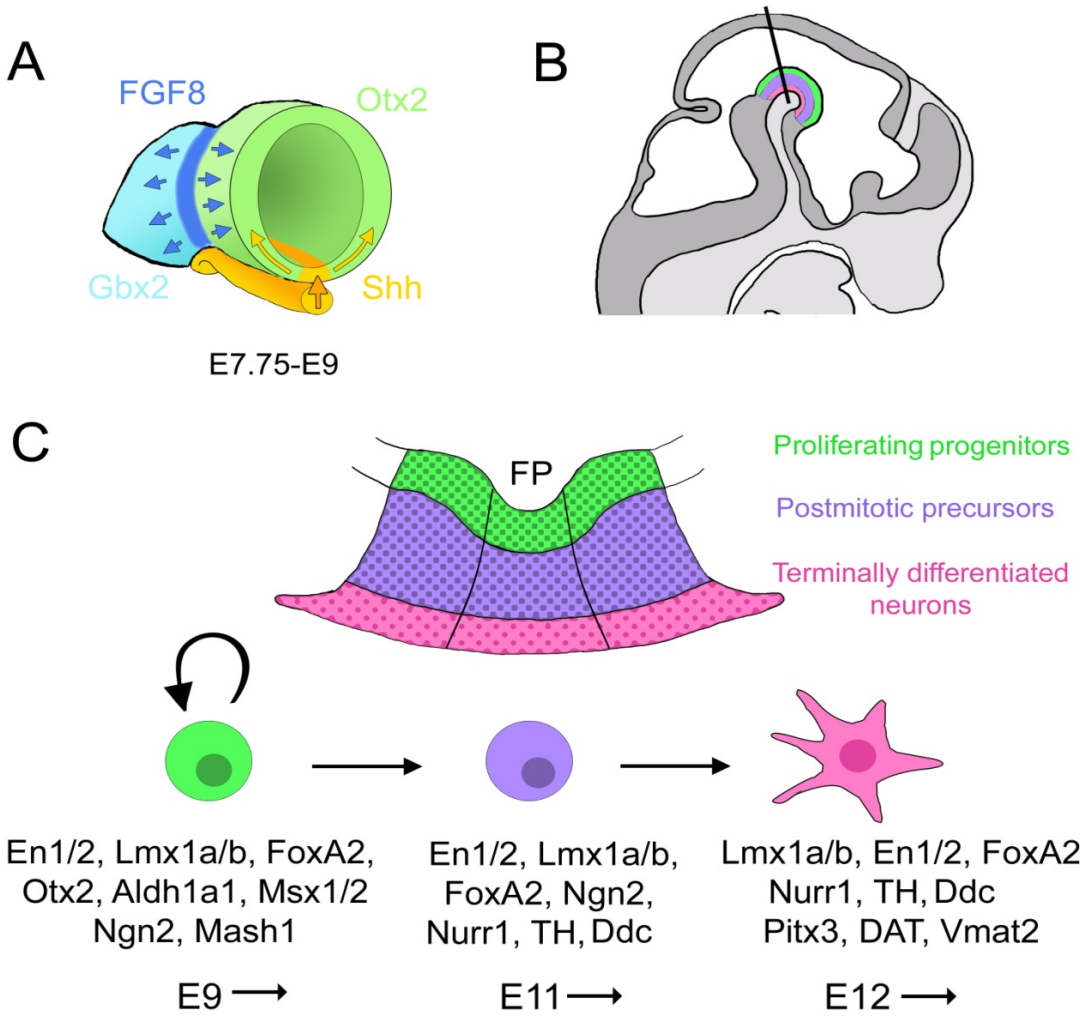

Figure 12. Development of midbrain dopaminergic neurons.

(**A**) Cross section of a neural tube depicting midbrain-hindbrain region. Early specification events determine the dopaminergic domain in the ventral midbrain. Otx2 specifies the midbrain territory and gives it competence to respond to FGF8 from the isthmic organizer and Shh from the node and floorplate. Redrawn and modified from Gale and Li (2008). (**B**) Schematic view of sagittal E12.5 brain showing the location of meso-diencephalic dopaminergic domain. (**C**) Coronal view of the dopaminergic domain in the area depicted in (B). In the medio-lateral direction, the domain is divided into floor plate and lateral regions, which have somewhat different properties (see text). Color-coded model of dopaminergic neuron development, showing some of the factors expressed in proliferating progenitors (green), postmitotic but immature precursors (purple), and differentiated neurons (pink). Based on Smidt et al., (2003), Ang et al., (2006), Prakash and Wurst (2006), Smits et al., (2007), Smidt and Burbach (2007), Gale and Li (2008).

1.4.6.3.1. Regional specification

The induction of dopaminergic neurons requires secreted signals from floor plate and isthmic organizer (**Figure 12A**). These signals provide the ventral midbrain progenitors with competence to respond to a later expression of dopaminergic-specifying transcription factor Lmx1a (Andersson et al., 2006; Gale and Li, 2008).

Experiments with rat neural tube explants demonstrated that a combination of Shh and FGF8 is able to induce the formation of midbrain dopaminergic neurons (Ye et al., 1998). This does not happen in the hindbrain, implying that other factors must have primed the midbrain progenitors to react to these signals correctly. Later it was shown that this priming happens via midbrain-specifying transcription factor Otx2 (reviewed in Smidt and Burbach, 2007). Shifting the caudal border of midbrain anteriorly or posteriorly, via manipulation of *Otx2* expression, results a concomitant shift of dopaminergic domain. Anterior shift of *Otx2* changed the former caudal midbrain into rostral hindbrain, where serotonergic neurons then were able to develop. Otx2 also represses *Shh*, possibly via *FoxA2*, and thus regulates D-V patterning of the midbrain (Simeone et al., 2011).

In chick, TGB-beta signaling induces *Shh* and is thus required for the induction of midbrain dopaminergic neurons (Farkas et al., 2003). Although a similar inductive role has not been demonstrated in mice, TGF-beta and TGF-alpha are both required for the maintenance of dopaminergic neurons both in chick and in mice (Blum., 1998; Farkas et al, 2003; Roussa et al., 2004; Roussa and Krieglstein, 2004). Also FoxA1 and FoxA2 regulate Shh signaling in the ventral midbrain (Mavromatakis et al., 2011).

1.4.6.3.2. Proliferation and neurogenesis

The first dopaminergic progenitors can be detected in the ventral midbrain at E9.5 by their expression of *Aldh1a1* (Haselbeck et al., 1999; Westerlund et al., 2005; **Figure 12C**). Later *Aldh1a1* expression is restricted to a subset of dopaminergic neurons (McCaffrey and Drager, 1994; Wallen et al., 1999; Chung et al., 2005a). Proliferating dopaminergic progenitors express several transcription factors, none of which is completely specific to dopaminergic neurons. These factors, which are involved in fate specification, suppression of alterate fates, and progression of neurogenesis, include *Otx2*, *Lmx1a/b*, *Msx1a/b*, *Ngn2*, *Mash1*, and *En1/2* (Ang, 2006; **Figures 12C** and **13**).

Midbrain VZ expresses *En1/2* widely around E9.5-E11.5. En1/2 are known to maintain the isthmic organizer, and are required for the survival of postmitotic dopaminergic neurons (see below). However their function specifically in proliferating dopaminergic progenitors has not been addressed. It is possible that they are involved in the general patterning of this domain.

Otx2 is involved in the patterning of dopaminergic domain, but also in neurogenesis (Vernay et al., 2005; Prakash et al., 2006). Inactivation of *Otx2* in neuronal progenitors results in the loss of *Ngn2* and *Mash1*, reducing the number of dopaminergic neurons (Vernay et al., 2005). However, terminal differentiation in these neurons is not compromised, as they are still TH^+ and $Pitx3^+$.

Lim homeodomain containing transcription factors *Lmx1a* and *Lmx1b* are expressed in the dopaminergic domain of the ventral midbrain (Andersson et al., 2006; Smidt et al., 2000). *Lmx1a* expression in the ventral midbrain begins later than *Lmx1b,* but later both of these genes become co-expressed, and continue to be expressed also in mature dopaminergic neurons (Andersson et al., 2006; Ono et al., 2007; Deng et al., 2011).

These two genes appear to have partially redundant functions in the dopaminergic neuron specification (Yan et al., 2011). *Lmx1a* is able to fully compensate for the loss of *Lmx1b*, but not vice versa (Yan et al., 2011). Together with FoxA2 and FoxA1, Lmx1a/b promote neurogenesis of dopaminergic neurons and suppress alternate neuronal fates (Chung et al., 2009; Nakatani et al., 2010; Lin et al., 2009; Yan et al., 2011; **Figure 13**). Forced expression of *Lmx1a* in mouse ES cells converts them efficiently into dopaminergic fate, if *Shh* is also present (Andersson et al., 2006).

Lmx1a/b target, transcription factor Msx1, suppresses alternate neuronal fates of dopaminergic progenitors by r epressing *Nkx6.1*, and induces the acquisition of neurogenic potential via *Ngn2* (Andersson et al, 2006). The neurogenesis is then driven forward by *Ngn2* and *Mash1* (Kele et al., 2006). Although *Mash1* and other genes are partially able to compensate for the loss of *Ngn2*, the majority of the dopaminergic neurons in *Ngn2* mutants are lost. After becoming postmitotic, dopaminergic neurons soon downregulate *Ngn2* expression.

Although *Shh* is required for the early induction of dopaminergic neurons, later it needs to be downregulated in the dopaminergic domain to allow proliferation and neurogenesis (Joksimovic et al., 2009b). This is accomplished by Wnt-signaling (Joksimovic et al., 2009b). Wnt1, which is expressed in the ventral midbrain midline, controls the proliferation of dopaminergic progenitors by positively regulating *CyclinD1* (Panhuysen et al., 2004; Yan et al., 2011; **Figure 13**). Loss of *Wnt1* impairs also the induction and differentiation of the dopaminergic neurons (Prakash and Wurst, 2006a; Prakash et al., 2006).

However, in mouse mutants in which *Wnt1* is lost also in the isthmus, these defects may be secondary due to downregulation of isthmic *Fgf8*. To circumvent the impairment of isthmic organizer, Wnt-signaling has been inactivated using Shh-Cre. Shh-Cre-mediated inactivation of *beta-catenin* leads to upregulation of *Shh*, and reduction of neurogenesis in the floor plate (Joksimovic et al., 2009b). Thus, *Wnt* expression in the dopaminergic domain antagonizes the anti-neurogenic effect of Shh, and permits dopaminergic neurogenesis. Supporting these observations, the inactivation of Wnt co-receptor *Lrp6* delays dopaminergic neurogenesis (Castelo-Branco et al., 2010). In contrast, the stabilization of *beta-catenin* by En1-Cre in the whole midbrain and rhombomere 1 expands the dopaminergic domain (Chilov et al., 2010).

1.4.6.3.3. Maturation, terminal differentiation, and survival

First postmitotic dopaminergic neurons in the ventral midbrain appear around E11.5, marked by the presence of TH (**Figure 12C**). At this stage, these precursors are still in an immature state and lack genes normally present in terminally differentiated neurons. These immature neurons express transcription factors which are involved in survival and acquiring the correct neurotransmitter identity, such as *En1/2*, *Lmx1a/b* and *Nurr1*. *Lmx1b* appears dispensable for dopaminergic neuron development (Yan et al., 2011). In contrast, Lmx1a can directly regulate expression of *Nurr1* (*Nr4a2*) and *Pitx3* (Chung et al., 2009). However, the loss of *Lmx1a* does not prevent the development of $Nurr1^{+}Pitx3^{+}$ neurons, although their number in the ventral midbrain is significantly reduced, apparently due to decreased neurogenesis (Ono et al., 2007; Deng et al., 2011). This is apparently due to the redundancy with Lmx1b, as the combined inactivation of both *Lmx1a* and *Lmx1b* by Shh-Cre leads to a drastic loss of dopaminergic neurons (Yan et al., 2011).

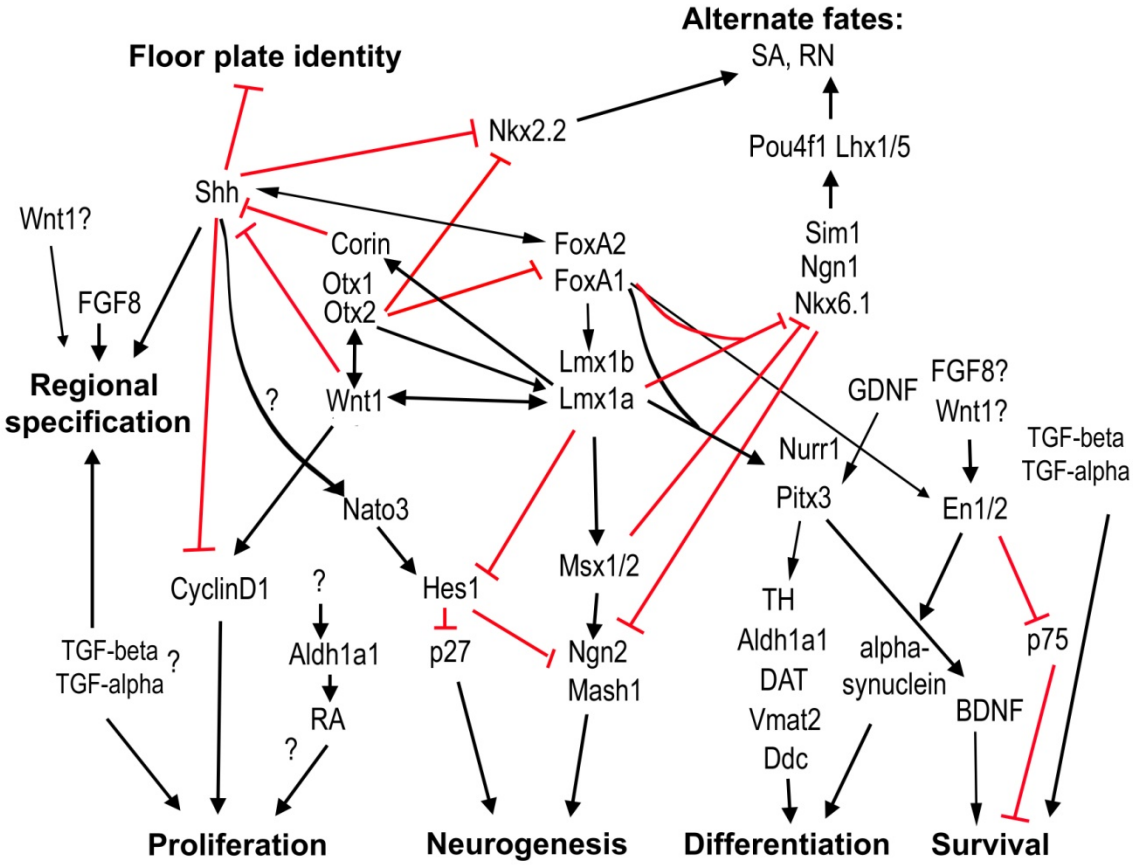

Figure 13. Genetic networks controlling the development of midbrain dopaminergic neuron development.

A simplified view of some of the genetic interactions which regulate different aspects of dopaminergic neuron development. Due to a limited space, all interactions and genes involved cannot be shown here. Lmx1a and Lmx1b are one of the most important transcription factors in this model. Together with FoxA factors Lmx1a/b cooperatively promote the differentiation of dopaminergic neurons via Msx1-Ngn2, and suppress alternate neuronal fates. Lmx1a/b also promote proliferation of dopaminergic progenitors via Wnt1, which regulates *CyclinD1* and inhibits *Shh*. Shh prevents neurogenesis and proliferation in the dopaminergic domain, and suppresses both floor plate identity and alternative neuronal fates. Nurr1 and Pitx3 activate together several genes which regulate neurotransmitter phenotype. En1/2 are required for differentiation and survival of dopaminergic neurons. Question marks indicate hypothesized activities which have not yet been demonstrated. SA, serotonergic; RA, retinoic acid; RN, red nucleus. Based on Ye et al., (1998); Simon et al., (2001); Farkas et al., (2003); Roussa et al., (2004); Andersson et al., (2006a,b); Ang et al., (2006); Jacobs et al., (2007); Chung et al., (2009); Joksimovic et al., (2009b); Li et al., (2009); Nakatani et al., (2010); Yan et al., (2011); Peng et al., (2011); Simeone et al., (2011).

En1 and *En2* are required for maturation and survival of postmitotic dopaminergic precursors. In *En1/2* mutants, TH^+ dopaminergic precursors fail to differentiate, indicated by the lack of Pitx3, and apoptotically die by E 14.5 (Simon et al., 2001; Simon et al., 2004). En1/2 may promote the dopaminergic neuron survival by suppressing *p75Ntr* expression (Alavian et al., 2009). As shown by c ell-mixing and RNAi experiments, this requirement for *En1/2* in survival appears to be cell-autonomous, and independent of the earlier expression in progenitors (Albéri et al., 2004). Engrailed factors are needed in dopaminergic neurons throughout life. *En1* heterozygous mice display a progressive loss of midbrain dopaminergic neurons and problems in motor coordination (Sgadò et al., 2006; Sonnier et al., 2007). In addition, *Engrailed-1* polymorphism in humans has been associated with Parkinson's disease (Haubenberger et al., 2011). En1/2 activate the transcription of *alpha-synuclein*, a gene linked to molecular pathology of Parkinson's disease (Simon et al., 2001). Furthermore, exogenous En1/2 are able to protect dopaminergic neurons in toxin-induced Parkinson models, and increase striatal dopamine levels (Alvarez-Fischer et al., 2011).

Orphan nuclear receptor *Nurr1* is expressed in postmitotic dopaminergic precursors, and its expression is maintained postnatally (Zetterström et al., 1996). However, *Nurr1* is not specific for midbrain dopaminergic neurons but it is widely expressed in other parts of CNS. Recently it was shown that Nurr1 in astrocytes and microglia protects dopaminergic neurons from pro-inflammatory neurotoxic mediators (Saijo et al., 2009). Nurr1 can form a dimer with RXR, suggesting a convergence with retinoic acid signaling (Perlmann and Jansson, 1995). It also regulates the differentiation and survival of dopaminergic neurons in a direct interaction with p57 (Joseph et al., 2003).

In the dopaminergic neurons Nurr1 is required for the maintenance of *Th* expression (Zetterström et al., 1997; Saucedo-Cadenas et al., 1998). In *Nurr1* mutant embryos, dopaminergic neurons begin to develop and differentiate normally, but fail to maintain *Th* expression and are subsequently lost (Le et al., 1999). Besides *Th*, Nurr1 regulates the expression of several genes which are involved in dopamine synthesis and secretion pathway, and expressed in terminally differentiated dopaminergic neurons. These include *dopa decarboxylase (Ddc,* also known as *Aadc), vesicular monoamine transporter 2 (Vmat2)*, *Aldh1a1*, *dopamine transporter (DAT)*, and *Ret* (Le et al., 1999; Zetterström et al., 1997; Wallen et al., 1999; Wallen et al., 2001; Smits et al., 2003; Jacobs et al., 2009a).

Pitx3 is a specific marker of maturing midbrain dopaminergic neurons (Maxwell et al., 2005). During early embryogenesis, dopaminergic precursors in the ventral tegmental region express first *Th* and then *Pitx3*, whereas laterally locateted SNpc precursors express first *Pitx3* and then *Th*.

In *Pitx3* ^{null} mice, only a subset of dopaminergic neurons are affected, mainly in SNpc (Nunes et al., 2003; Smidt et al., 2004; Hwang et al., 2003). Indeed, it has been reported that *Pitx3* polymorphism is associated with early onset form of Parkinson's disease and schizophrenia (Bergman et al., 2010a,b). One of the targets of Pitx3 is *Aldh1a1* (Chung et al., 2005a; Jacobs et al., 2007). Retinoic acid treatment of *Pitx3* ^{null} mice was able to rescue TH expression in the SNpc neurons (Jacobs et al., 2007). Pitx3 is thought to induce the expression of *brain derived neurotrophic factor (Bdnf)* and *glial cell line derived neurotrophic factor (Gdnf)*, which promote the survival of dopaminergic neurons (Li et al., 2009). However, more recent findings indicate that GDNF acts upstream of Pitx3, which in turn induces *Bdnf* expression in SNpc (Peng et al., 2011).

Nurr1 requires the presence of Pitx3 for its full activity in regulating target genes, such as *Th* (Jacobs et al., 2009b). In fact, Pitx3 and Nurr1 might form a complex, thus regulating the expression of many of their target genes together (Jacobs et al., 2009a,b; Hwang et al., 2009).

2. AIMS OF THE STUDY

In this study, we aimed to analyze how isthmic FGF signaling regulates the development of the embryonic mouse midbrain and hindbrain. Our specific interests have been the regulation of neurogenesis, and the development of various nuclei in this region, especially dopaminergic neurons. As our main approach, we conditionally inactivated *Fgfr1-3* in various combinations specifically in the developing midbrain-hindbrain region.

Our specific aims were:

1. Identifying novel FGF signaling targets, which might regulate the development of the midbrain and hindbrain, and analyzing their expression patterns.

2. Analyzing neurogenesis in conditional *Fgfr1* mutant embryos.

3. Studying whether FGFR1-3 can cooperatively respond to isthmic FGF signals, and how these receptors regulate cell survival, proliferation, patterning, and neurogenesis in the midbrain and hindbrain.

4. Assessing the role of FGF signaling in the regulation of proliferation vs differentiation decisions in neuronal progenitors.

5. Studying the function of FGF signaling in the development of serotonergic as well as mesodiencephalic dopaminergic neurons.

3. MATERIALS AND METHODS

3.1. Materials

Mouse strains used in this study and their references are listed in **Table 1**. PCR primers used for genotyping the mice can be found in the original references. Conditional *Fgfr* alleles, containing LoxP sites (*flox*), were mainly inactivated by En1-Cre, which is active in the midbrain and rhombomere 1 from E8.5 onwards (**Figure 14**).

Mutant embryos were always compared to their littermate controls, which in articles I-III are referred to as "wild-type", and in IV as "control". In Studies I and II, the control embryos were Cre-negative and did not contain an *Fgfr3null* allele (II). In III-IV, the control embryos were either Cre-negative, containing unrecombined forms of *Fgfr flox* alleles; or had recombined *Fgfr1flox* allele as heterozygous; recombined *Fgfr2flox* allele as hetero- or homozygous; and *R26R*. In analyses involving quantification, control embryos were Cre-negative. In all analyses, the phenotype of the control embryos was indistinguishable from true wild-type (ICR or NMRI) embryos. In all experiments and analyses, $n \geq 3$ (for a more detailed description, see Methods in each article).

Mouse strain	Description	Reference	Used in
Fgfr1flox	Conditional allele of *Fgfr1*	Trokovic et al., 2003	I-IV
Fgfr2flox	Conditional allele of *Fgfr2*	Yu et al., 2003	II-IV
Fgfr3null	Null allele of *Fgfr3*	Colvin et al., 1996	II-III
Fgfr1IIICn	Null allele of *Fgfr1*	Partanen et al., 1998	III-IV
En1-Cre	*Cre-recombinase* under *Engrailed1*-promoter	Kimmel et al., 2000	I-IV
Shh-Cre	*Cre-recombinase* under *Sonic hedgehog* promoter	Harfe et al., 2004	III-IV
Th-Cre	*Cre-recombinase* under *tyrosine hydroxylase* promoter	Lindeberg et al., 2004	IV
DAT-Cre	*Cre-recombinase* under *Dopamine transporter* promoter	Ekstrand et al., 2007	IV
Fst null	*Follistatin* null mutant	Matzuk et al., 1995	I
R26R	Mouse-line carrying inducible *beta-galactosidase*	Soriano, 1999	III-IV

Table 1. Mouse strains.

3.2. Methods

Methods and their references are shown in **Table 2**.

Antibodies used in immunohistochemistry (IHC), and *in situ* hybridization (IHS) probes used in the Studies I-IV are provided in the Methods of each article/manuscript, and additionally in Kala et al., (2009). In addition, X-gal staining in Figure 14 was performed as previously described (Kala et al., 2008).

Method	Described in	Used in
ISH on sections	Wilkinson and Green, 1990, IV	I-IV
Whole-mount ISH	Henrique et al., 1995	I-II
PCR genotyping	Trokovic et al., 2003	I-IV
TUNEL assay on sections	Trokovic et al., 2005	II
TUNEL on whole embryos	Chi et al., 2003	II
Microarray analysis	I	I
BrdU incorporation	Trokovic et al., 2005	II-III
BrdU+EdU incorporation	III	III
Statistical similarity analysis	Rita and Ekholm, 2007	III
IHC on sections	I, III	I-IV
Pair-cell assay	Shen et al., 2002	III
Retinoic acid treatment	Jacobs et al., 2007	IV
Striatal dopamine measurement	Airavaara et al., 2006	IV
Neurofilament staining	Trokovic et al., 2003	II
Electron microscopy	III	III
Semi-thin sections	Trokovic et al., 2003	II
Generation of chimeric embryos	Nagy et al., 2002, III	III, IV

Table 2. Methods.

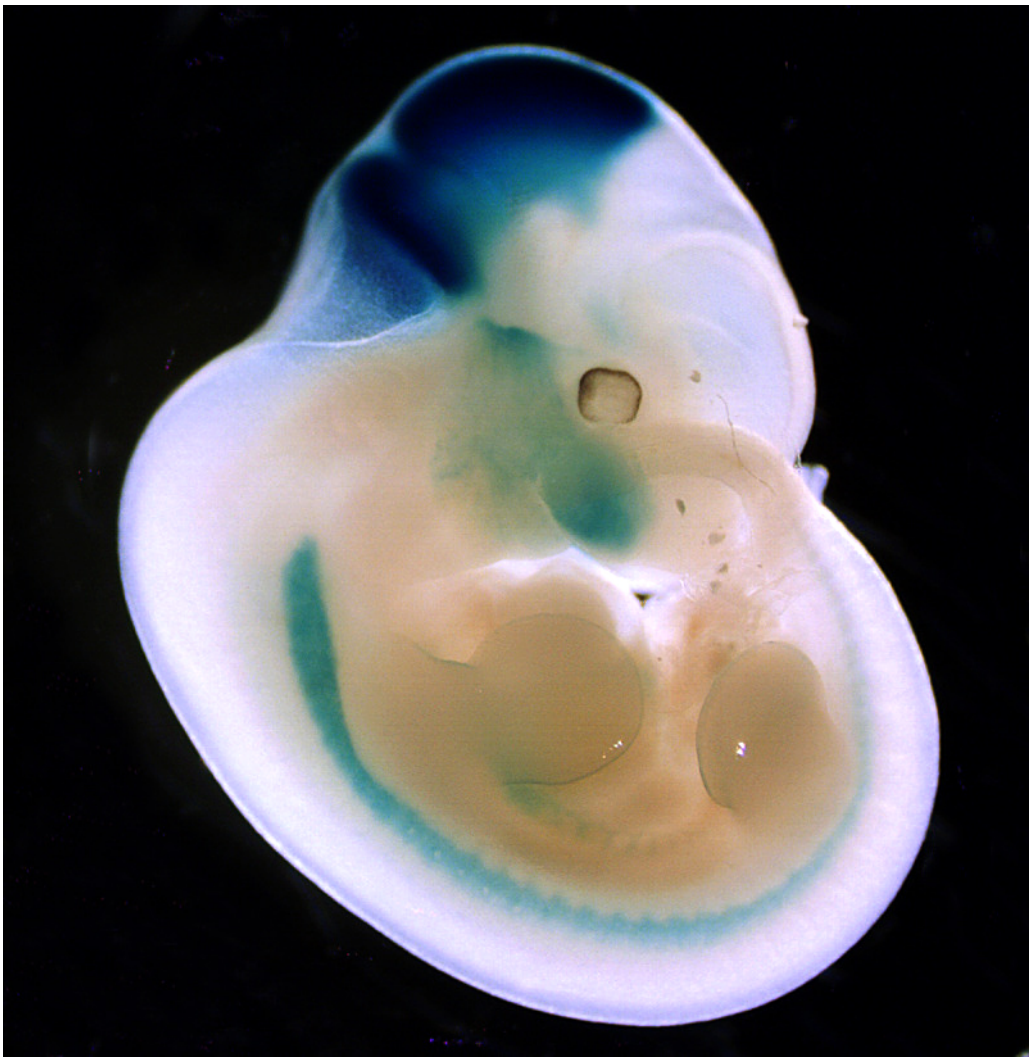

Figure 14. En1-Cre activity region.
X-gal-stained E11.5 mouse embryo which contains an En1-Cre-recombined *R26R* allele, expressing *betagalactosidase*. In the developing brain, En1-Cre efficiently recombines *R26R* in the entire midbrain and anterior hindbrain. Its activity in the midbrain and hindbrain begins around E8.5 (Trokovic et al., 2003). Recombination can also be seen in the spinal cord and neural-crest-derived cells in the head region.

4. RESULTS

4.1. Gene expression and neurogenesis in *Fgfr1*^{cko} midbrain-hindbrain region (I)

Previous work in our group had demonstrated that *Fgfr1* is required for the development of posterior midbrain and anterior hindbrain (Trokovic et al., 2003). *Fgfr1*^{cko} embryos, in which *Fgfr1* is conditionally inactivated in the midbrain-hindbrain region using En1-Cre, fail to form a coherent midbrain-hindbrain boundary, and consequently lose the signaling center isthmic organizer. This results in the loss of dorsal structures: cerebellar vermis and inferior colliculus. Because FGF8, together with Shh, had been shown to induce the development of both dopaminergic and serotonergic neurons *in vitro*, the loss of FGFR1-mediated signaling might affect these neuronal population also *in vivo* (Ye et al., 1998). According to Trokovic et al., (2003) all major nuclei in midbrain and hindbrain region appeared to be present in postnatal animals. However, these first analyses of *Fgfr1*^{cko} embryos lacked the analysis of serotonergic neurons, and a more detailed characterization of midbrain dopaminergic neurons. In this study, we analyzed the role of FGFR1-mediated signaling in the development of these two neuronal subgroups.

In addition, we wanted further understand the function of FGF signaling in the midbrain and hindbrain. This involves identifying new FGF-regulated genes which are involved in the development of this brain region.

4.1.1. Gene expression profiling of *Fgfr1*^{cko} mutant embryos

Here we used an Affymetrix cDNA microarray approach to compare gene expression changes between E10.5 wild-type and *Fgfr1*^{cko} midbrain-hindbrain tissue. This embryonic stage was chosen, because the expression of most known FGF targets between wild-type and mutant samples was assumed to show a clear difference by then. In addition, the larger size of the embryos compared to E9.5 provided more material for the mRNA extraction. Two pools of both wild-type and mutant samples (n = 5-6 in each pool; individual samples pooled after the genotypes had been confirmed by PCR) were compared in a total of four data sets. The genes chosen for further study were the ones below 0.75 f oldchange threshold for downregulated genes, or above 1.41 f or upregulated genes in at least 3 out of 4 i ndividual comparisons. Altogether 51 downregulated genes and 20 upr egulated genes passed these thresholds. We then validated the results by *in situ* hybridization for 25 downregulated and 15 upregulated genes.

4.1.2. Downregulated genes

Based on t heir expression pattern in midbrain-rhombomere 1, a s well as on t heir response to the loss of FGFR1-mediated signaling, the downregulated genes can be divided into three groups (I, Table 1, Figs. 1, 2). First, genes which were restricted to a narrow cell population in the midbrain-hindbrain boundary in the wild-type. These genes were completely lost in *Fgfr1*^{cko} embryos. Second, genes which displayed a wider expression gradient around the midbrain-hindbrain boundary in the wild-type, and only

residual ventral expression in mutants. Third, genes which in the wild-type were expressed throughout the midbrain but absent in a narrow area in the isthmus, and in mutants were entirely downregulated dorsally.

The first group included a negative cell-cycle regulator *Jumonji* (Toyoda et al., 2003); a positive FGF signaling regulator *Flrt3* (Bottcher et al., 2004); as well as *Trh*, *Mrp4,* and *Igfbp5,* whose functions in the brain development are currently unclear. In *Fgfr1*^{cko} midbrain they were absent (I, Figs. 1, 2, Supplementary Figs. 4, 5). The localization of these genes partially corresponded to the FGFR1-dependent, slowly proliferating boundary cells which disappear early in *Fgfr1*^{cko} embryos (Trokovic *et al.,* 2005).

The second group contained members of the *Fgf8* synexpression group, such as patterning genes *Pax5* and *En1/2*, as well as ligands, modulators and targets of the FGF signaling pathway, such as *Fgf8/17/18*, *Spry1/2*, *Dusp6* (*Mkp3), Erm* and *Pea3* (I, Figs. 1 and 2). In addition, *Canopy1*, whose expression resembles that of *En1* and *En2*, was identified as an FGF target in the midbrain region (I, Fig. 2, S 3). In the ventral midbrain, *Canopy1* expression appears to colocalize with *En1/2* in dopaminergic precursors (our unpublished data). The patch of residual expression which remained in the basal plate for most of these genes, may result from FGFR2 and FGFR3 compensating for the loss of FGFR1.

The third group included a member of the tumor necrosis factor receptor *Tnfrsf19*; as well as genes associated with Wnt signaling, such as *Tcf7*, *Drapc1,* and *Sfrp2* whose downregulation in *Fgfr1*^{cko} embryos likely resulted from the loss of isthmic *Wnt1* (I, Fig. 2). *Drapc1,* orthologous to human Wnt signaling target *APCDD1* (Takahashi et al., 2002), was identified as a novel mouse gene, and its expression pattern was published separately (Jukkola et al., 2004). More interestingly, the third group included *CyclinD2* and *Sox3,* which are involved in the regulation of cell cycle progression and neuronal stem cell maintenance, respectively (Bylund et al., 2003. Their downregulation in *Fgfr1*^{cko} embryos suggested decreased proliferation and increased neuronal differentiation.

4.1.3. Upregulated genes

Genes upregulated in *Fgfr1*^{cko} embryos (I, Table 2) included genes associated with neuronal differentiation, such as *vitronectin (Vtn)*, *Rgma* and *Ngfr (p75Ntr)*; a growth inhibitor *Wfdc1 (ps20)*; a regulator of both neural crest migration and interkinetic nuclear migration *Ednrb* (Wechsler-Reya, 2001; Matsunaga et al., 2006; Diolaiti et al., 2007; Nishikawa et al., 2011); and several genes whose function in vertebrate brain development is unknown, such as *Uncx4.1*, *Mab21l1*, and *Dach1*. They all displayed similar expression patterns in the E10.5 wild-type midbrain and rhombomere 1 – the expression was detected as a gradient in dorsal or ventral regions, or both, diminishing towards the isthmus (I, Fig 3). In *Fgfr1*^{cko} embryos these gradients extended across the midbrain-hindbrain boundary. Out of these genes, *Rgma* overexpression has been shown to result in increased neuronal differentiation in chick midbrain and hindbrain (Matsunaga et al., 2006).

In addition, some expressed sequence tags (ESTs) were discovered among the upregulated genes. Of these, the sequence of EST BM932503 (Affymetrix probe 137358_at) matched to the transcription factor *Pou2f2*, whose expression in the midbrain has not been characterized previously. We discovered that it was expressed in both tectum and ventral midbrain, as well as in the lateral rhombomere 1. EST

NM_028263, which was identified as *FGF binding protein 3 (Fgfbp3)* was the only gene directly related to FGF signaling found among the upregulated ones. It was expressed in the basal plate of rhombomere 1 and midbrain, and absent in the dorsal regions. *FGF binding protein 1* is expressed widely during embryogenesis, although mainly in non-neuronal tissues, and has been suggested to modulate the interaction of FGFs with their receptors (Abuharbeid et al., 2006; Aigner et al., 2002). In contrast to *Fgfbp1*, *Fgfbp3* was mainly expressed in the developing CNS. However, no reports of its function in the CNS development exist.

4.1.4. Midbrain-hindbrain nuclei appear normal in *Fst* mutant embryos

The gene displaying most upregulation in the microarray was BMP-antagonist *Fst*, whose fold change was 4.00. In wild-type embryos, *Fst* was expressed in dorsal midbrain and rhombomere 1 at E9.5, and already at this stage the upregulation was visible in the $Fgfr1^{cko}$ mutant hindbrain (I, Fig. 3 M , M'). In E10.5 mutants, *Fst* expression domains in both alar and basal plates of midbrain and r1 encompassed the entire boundary area. It has been shown in chick that isthmic FGF8 negatively regulates *Fst* expression (Alexandre et al., 2006). Fst in turn inhibits the activity of activin, which modulates roof plate development. Furthermore, BMP-signaling regulates the development of locus coeruleus (Vogel-Höpker and Rohrer, 2002). However, $Fst^{null/null}$ embryos showed no obvious changes in either brain morphology, dopaminergic and serotonergic neurons, or locus coeruleus (I, Fig. 4).

4.1.5. Increased neurogenesis in the $Fgfr1^{cko}$ midbrain-hindbrain boundary region

In midbrain, neurons differentiate in an anterior to posterior direction (LaVail and Cowan, 1971a,b). It was previously known that cells in the midbrain-hindbrain boundary area differentiate later than the cells located further away (Hirata et al., 2001; Trokovic et al., 2005;). Our *in situ* results, with genes either losing or extending their gradient at the boundary region, suggested an increased neurogenesis in $Fgfr1^{cko}$ mutants. We studied this by analyzing the expression of Notch-effector *Hes3*, known to repress neurogenesis; transcription factor *Ngn2*, which in turn promotes neurogenesis; and a marker of postmitotic neurons, Tuj1, in $Fgfr1^{cko}$ midbrain. Whereas *Hes3* was downregulated, *Ngn2* expression and $Tuj1^{+}$ domain expanded towards the boundary region (I, Fig 5). This suggests that near the midbrain-hindbrain boundary FGFR1-mediated signaling normally suppresses neurogenesis.

4.1.6. The loss of FGFR1-mediated signaling does not affect the survival of dopaminergic neurons and locus coeruleus

Because we could observe increased neurogenesis is $Fgfr1^{cko}$ midbrain, we wanted to inspect more closely the various nuclei developing in the midbrain and hindbrain region. Nuclei of III and IV cranial nerve were able to develop in mutants, but appeared fused together, whereas locus coeruleus cells were more scattered compared to the wild-type (I, Fig. 5, 7). These observations support earlier findings from newborn and adult $Fgfr1^{cko}$ mice (Trokovic et al., 2003).

To investigate how the loss of FGFR1-mediated signaling affects dopaminergic neurons, we analyzed the expression of genes participating in their development, such

as *Nurr1*, *Aldh1a1* (here called *Aldh1*) and *Pitx3*. In E10.5 and E11.5 mutants, the expression domains of these genes were expanded caudally, likely following the expansion of *Otx2* domain (I, Fig. 6). Consequently at E15.5, when all midbrain dopaminergic neurons are postmitotic and TH^+, we could detect a caudal shift in their position. In the wild-type, TH^+ cells were located throughout the midbrain and caudal diencephalon, whereas in $Fgfr1^{cko}$ mutants they were mostly located in the caudal midbrain. However, the total number of dopaminergic neurons in mutants was not markedly altered. In addition, the caudal boundary of *Otx2* had become more diffuse in mutants by E15.5, suggesting that ventral midbrain and rhombomere 1 cells may be mixing and thus forming a tissue mosaic in the caudal midbrain.

4.1.7. Rostral serotonergic neurons fail to develop in $Fgfr1^{cko}$ mutants

Next, we turned our attention towards serotonergic raphe nuclei in rhombomere 1. In E15.5 mutants, a part of the dorsal raphe nucleus was lost, which reduced the total number of serotonergic neurons (I, Fig. 7). In fact, the disappearance of the most rostral serotonergic precursors was detected already in E10.5 and E11.5. At this time, these cells normally express *Gata3*, *Mash1* and *Pet1*, which in $Fgfr1^{cko}$ mutants were downregulated. Importantly, *Otx2* was not expressed in the area which displayed the downregulation of serotonergic neuron genes, which confirmed that this region still had rhombomere 1 identity.

4.1.8. Summary

The Affymetrix microarray is a valid approach to study FGF regulated gene expression changes in the developing midbrain and hindbrain, demonstrated by its ability to identify several known FGF target genes. In addition, the screen revealed novel genes regulated by FGF signaling, whose function in the midbrain-hindbrain development is mostly unknown. Based on the results from this study, we proposed a model in which FGF-signaling from the isthmus maintains two types of gene expression gradients in the developing midbrain and rhombomere 1. First, the gradients originating in the boundary area regulate both FGF-signaling, antero-posterior patterning, promote neuronal progenitor maintenance and proliferation, and suppress neurogenesis near the boundary region. Second, the opposing gradients, normally absent from the boundary region, are likely involved in neuronal differentiation. The loss of FGFR1-mediated signaling disturbs these gradients, and leads to premature neurogenesis in the midbrain and hindbrain and to the loss of proliferating neuronal progenitors in the boundary area. A-P patterning changes result in the caudal shift in the position of dopaminergic neurons, without affecting their survival. However, as the development of dorsal raphe nuclei was affected in mutants, the serotonergic neurons appear sensitive to the loss of isthmic FGF8. Thus, neuronal populations on either side of midbrain-hindbrain boundary appear to differ in their requirements for FGF-signaling from the isthmus.

4.2. Cooperation of FGF receptors in patterning, cell survival, and neurogenesis in the midbrain and hindbrain (II)

Most FGF signaling pathway components showed residual expression in ventral midbrain of *Fgfr1*^{cko} mutants, and the midbrain-hindbrain phenotype in these mutants was much milder than in *Fgf8*^{cko} embryos, although same En1-Cre had been used to inactivate both alleles (Chi et al., 2003). This implied that other FGF-receptors in the region were participating in the FGF signaling. Previous analyses had revealed that *Fgfr2* and *Fgfr3* are expressed in the developing mouse midbrain and rhombomere 1, but they are absent from the boundary region (Liu et al., 2003; Trokovic et al., 2005; Blak et al., 2005). On the other hand, *Fgfr2*^{cko} or *Fgfr3*^{null}, or *Fgfr2*^{cko}*;Fgfr3*^{null} compound mutants have a normal brain phenotype (Blak et al., 2007), so it was assumed that FGFR1 was the main receptor receiving isthmic FGF8 signal. In this work, we investigated the possible cooperation of FGFR1, FGFR2, and FGFR3 in patterning, regulation of cell survival, proliferation and neurogenesis, as well as in the development of various nuclei in the midbrain and rhombomere 1, s uch as dopaminergic and serotonergic neurons. For this, we generated compound mutants carrying different combinations of En1-Cre-inactivated *Fgfr1*^{cko}, *Fgfr2*^{cko} as well as *Fgfr3*^{null} alleles. The term "*Fgfr* compound mutants" refers collectively to *Fgfr1*^{cko}*;Fgfr2*^{cko} and *Fgfr1*^{cko}*;Fgfr2*^{cko}*;Fgfr3*^{null} embryos. These embryos lacked *Fgfrs* in the entire midbrain and rhombomere 1. The inactivation of conditional *Fgfr1* and *Fgfr2* alleles in the midbrain was verified with ISH using probes which recognize the floxed region (II, Supplementary Fig. S1).

4.2.1. General brain morphology in *Fgfr* compound mutants

First we wanted to compare the brain morphology between the different *Fgfr* compound mutants. If FGFRs were indeed cooperating in the midbrain and hindbrain, the inactivation of all three receptors should produce the most severe phenotype, corresponding to *Fgf8*^{cko} embryos. The brain morphology of *Fgfr1*^{cko}*;Fgfr3*^{null} embryos was similar to *Fgfr1*^{cko} mutants – smaller inferior colliculi, and absent vermis of the cerebellum (II, Fig 1 and Table 1). In contrast, the phenotypes of *Fgfr1*^{cko}*;Fgfr2*^{cko} and *Fgfr1*^{cko}*;Fgfr2*^{cko}*;Fgfr3*^{null} brain were more severe – the superior colliculi were now also absent, and cerebellum was unable to develop. Morphologically, the brain phenotype of *Fgfr1*^{cko}*;Fgfr2*^{cko}*;Fgfr3*^{null} embryos resembled that of *Fgf8*^{cko} embryos, suggesting that all three receptors indeed participated in receiving FGF8 from the isthmus. Both *Fgfr1*^{cko}*;Fgfr2*^{cko} and *Fgfr1*^{cko}*;Fgfr2*^{cko}*;Fgfr3*^{null} mice died at birth.

4.2.2. A-P patterning defects and apoptosis in the dorsal midbrain

To study the cause for the abnormal brain morphology in more detail, we analyzed FGF targets, patterning genes and apoptosis in the early *Fgfr* compound mutant embryos. FGF ligands and targets, such as *Fgf8*, *Fgf17*, *Spry1*, *Erm* and *Pea3* showed downregulation in dorsal areas already at E9.5 – a day earlier than in *Fgfr1*^{cko} embryos (II, Fig.1). The downregulation of FGFs and their targets in ventral region was more prominent in compound mutants than in *Fgfr1*^{cko} embryos. For example, E9.5 *Fgfr1*^{cko}*;Fgfr2*^{cko} embryos displayed only a small ventral patch of *Fgf8* expression – a drastic change to only a slight downregulation observed in *Fgfr1*^{cko} isthmus. In the

Fgfr1^{cko};*Fgfr2*^{cko};*Fgfr3*^{null} embryos of the same age, *Fgf8* was entirely absent. This provides further support that all three receptors receive isthmic FGFs, and FGFR2 and FGFR3 are able to partially compensate for the loss of FGFR1.

In *Fgf8*^{cko} mutants, the loss of isthmic signals leads to apoptosis in the dorsal midbrain and anterior hindbrain in an early stage (Chi et al., 2003). Because the phenotype of especially *Fgfr1*^{cko};*Fgfr2*^{cko};*Fgfr3*^{null} embryos resembled that of *Fgf8*^{cko} embryos, we expected to see a similar phenomenon in them. Indeed, compared to the wildtype embryos, the midbrain-hindbrain region of *Fgfr* compound mutants appeared smaller already by E9.5. To investigate if this could result from increased cellular death, we TUNEL-stained E9.0 mutant embryos, as well as analyzed the tissue structure using semi-thin sections (II, Fig. 2). Whereas cell death was only mildly increased in the ventral side, we could observe an over two-fold increase in the number of TUNEL$^+$ cells in the dorsal regions. Consequently, locus coeruleus which develops in the dorsal rhombomere 1 was lost in mutants (II, Fig. 4, and Supplementary Fig. S2).

The loss of isthmic signaling might shift anterior and posterior boundaries of midbrain and rhombomere 1 (Irving and Mason, 2000; Scholpp et al., 2003). We analyzed A-P patterning changes in *Fgfr1*^{cko};*Fgfr2*^{cko} embryos (II, Fig. 3). In mutants, *Otx2* expression domain in midbrain expanded towards rhombomere 1, whose size was consequently reduced. This indicated a partial rhombomere 1- to - midbrain transformation. Similarly to *Fgfr1*^{cko} mutants, serotonergic neurons in the dorsal raphe nuclei were absent in *Fgfr* compound mutants (II, Fig. S2). The III and IV cranial nerves were also lost in mutants (II, Supplementary Fig. S2).

Although in E9.5 mutants the diencephalic *Pax6* expression showed no caudal shift, two days later the wild-type tissue from the diencephalon had replaced the dead tissue in the dorsal midbrain (data not shown). This caudal broadening of diencephalic region in mutants is also manifested by the expansion of posterior commissure (II, Fig. 1).

4.2.3. Midbrain dopaminergic neurons begin to develop but are lost by birth

In *Fgf8*^{cko} embryos, midbrain dopaminergic neurons are lost by E18.5 (Chi et al., 2003). In *Fgfr1*^{cko} midbrain, dopaminergic neurons displayed a shift towards caudal midbrain, but were able to survive. To study whether the loss of *Fgfr1*, *Fgfr2* and *Fgfr3* could together produce a more severe phenotype, we analyzed the number of TH$^+$ positive neurons in the compound mutants, in various stages of development.

Compared to *Fgfr1*^{cko} mutants, the dopaminergic neurons in *Fgfr1*^{cko};*Fgfr2*^{cko} and *Fgfr1*^{cko};*Fgfr2*^{cko};*Fgfr* ^{null} midbrain were clearly affected. At E12.5, the number of TH$^+$ cells in the ventral midbrain was reduced in *Fgfr* compound mutants (II, Fig. 4). By E15.5, only few TH$^+$ cells remained in mutants, and by birth, all TH immunoreactivity in the mutant midbrain was lost. In addition, the *Fgfr* compound mutant TH$^+$ cells failed to express *dopamine transporter (DAT)* and maintain *Pitx3*, both identifiers of mature DA neurons. At E12.5, the number of proliferative dopaminergic progenitors (Lmx1a$^+$ HuC/D$^-$) was also reduced (II, Fig. S2). In addition, *Aldh1a1* (in this paper, called *Aldh1*) expression in dopaminergic progenitors was downregulated in *Fgfr* compound mutants, and lost entirely by E11.5 (II, Fig. 5). Despite the loss of TH, *Pitx3* and *DAT*, dopaminergic progenitors showed no apparent defects in neurogenesis, as they continued to express *Ngn2*, *Lmx1a* and *Mash1* (II, Fig. 6). Also Wnt and Shh signaling pathways in the *Fgfr* compound mutant ventral midbrain remained normal by E11.5 (II, Fig. 7). Taken together, these results indicate that FGF-signaling affects both

proliferation and maturation of dopaminergic neurons. As some dopaminergic neurons were able to proliferate and develop even in *Fgfr1^{cko};Fgfr2^{cko};Fgfr3^{null}* embryos, but the maturation defect was visible already in *Fgfr1^{cko};Fgfr2^{cko}* embryos, the FGF signaling appears to be more critically required for the dopaminergic terminal differentiation than in their early proliferation.

4.2.4. Premature neurogenesis in the ventral midbrain

In *Fgfr1^{cko}* mutants, the downregulation of *Hes3*, *Sox3* and upregulation of Tuj1 indicated premature neuronal differentiation in the midbrain-hindbrain boundary. To investigate whether this effect was more prominent in *Fgfr* compound mutants, we analyzed cell proliferation and differentiation in the ventral midbrain.

Supporting the data from *Fgfr1^{cko}* embryos, cell-cycle regulators *CyclinD1* and *CyclinD2* were downregulated in *Fgfr* compound mutant midbrain (II, Fig. 8). This was especially clear in the dorsal midbrain, whereas the ventral expression remained rather normal in *Fgfr1^{cko};Fgfr2^{cko}* mutants – likely maintained by F GFR3. Indeed, *Fgfr1^{cko};Fgfr2^{cko};Fgfr3^{null}* embryos appeared to lack *CyclinD1* also ventrally.

Furthermore, the downregulation of *Sox3* was more evident in *Fgfr* compound mutants than in *Fgfr1^{cko}* embryos (II, Fig. 8 and 9; compare to I, Fig. 2). Interestingly, the level of Sox3, but not Sox2, was clearly decreased in the ventral midbrain. However, the Sox2^{+} layer of proliferative progenitors was narrower in the *Fgfr* compound mutants (II, Fig. 9). This was accompanied by a thicker layer of postmitotic neurons in the mantle zone. In fact, the first postmitotic neurons were visible already at E9.5 in *Fgfr1^{cko};Fgfr2^{cko};Fgfr3^{null}* embryos – almost one day earlier than the onset of neurogenesis in the wild-type. This premature neurogenesis became more evident as neuronal development proceeded, and by E11.5, the VZ in mutants had narrowed down to less than 50% of its normal size. This was not, however, majorly reflected in the proliferation of ventricular zone progenitors – measured by the ratio of BrdU^{+} to Sox2^{+} nuclei.

4.2.5. Summary

The experiments with *Fgfr* compound mutants allowed us to present a model on FGFR1-3 cooperation in the midbrain and rhombomere 1. At the midbrain-hindbrain boundary, only FGFR1 receives isthmic FGFs and supports both boundary cells, as well as serotonergic neurons in rhombomere 1. Further away from the isthmus, FGFR2 and FGFR3 cooperate with FGFR1 to support the identity of midbrain and rhombomere 1. More importantly, they promote cell survival in the dorsal regions, and maintain neuronal progenitors in the ventral regions, possibly acting through SoxB1 family members. In addition, FGF signaling appears to regulate terminal differentiation and survival of midbrain dopaminergic neurons.

4.3. Basal FGF8 gradient regulates neurogenesis in the developing midbrain via *Hes1* (III)

As we had detected postmitotic neurons appearing prematurely in *Fgfr* compound mutant midbrain, we next asked what the link is between FGF signaling and neuronal progenitor maintenance. The main factors known to contribute to the proliferation vs. differentiation balance in neuronal progenitors cells are SoxB1 family members Sox1-3, Notch-effectors in the Hes family, and their antagonists – Neurogenins, Mash1, and Dll1. In addition, the various properties of neuronal progenitors are known to tip the balance towards differentiation. These include a lengthening of the cell cycle; mitotic spindle positioning which would lead to uneven distribution of apical or basal membrane components; and the loss of apicobasal polarity. In this study, we analyzed these features in $Fgfr1^{cko};Fgfr2^{cko}$ embryonic neuronal progenitors. In addition, we aggregated wild-type (ICR) and $Fgfr1^{cko};Fgfr2^{cko}$ morulae to create chimeric embryos. Mutant cells in the midbrain-hindbrain region of these chimeras could be distinguished from the wild-type cells, as they were by betagalactosidase^{+}.

4.3.1. The loss of *Hes1* correlates with increased neurogenesis in the $Fgfr1^{cko};Fgfr2^{cko}$ ventricular zone

Hes1 prevents the progression of neurogenesis by repressing *Dll1*, *Mash1*, *Ngn2*, and *p57*, thus maintaining neuronal progenitors in an undifferentiated state (Kageyama et al., 2007). Analysis of *Hes1* expression in the ventral midbrain revealed that in mutants this gene was gradually downregulated (III, Fig. 1). Consequently, VZ showed an increase in the expression of proneural genes such as *Ngn2, Dll1*, and *Mash1*, and in the number of p57^{+} cells (III, Fig. 1 and Supplemental Fig. S1). Supporting this, mutant progenitors were also rapidly exiting the cell cycle (III, Fig. 2). We analyzed the most ventral region of the midbrain, which produces dopaminergic neurons (Lmx1a^{+}), separately from the more lateral regions (Lmx1a^{-}). The loss of *Hes1*, and the consequent increase in neurogenesis, occurred earlier in lateral regions compared to the ventral area. However, by E11.5 *Hes1* was also lost and proneural gene expression increased in the most ventral region. Furthermore, this effect of FGFR1/2-mediated signaling on neurogenesis appeared cell-autonomous (III, Figs. 3 and S2).

4.3.2. The progression of cell cycle, cell polarity and the orientation of cell division plane are not altered

Next, we wanted to know how the loss of FGF signaling affects the cell-biological properties of VZ progenitors. The length of the cell cycle, especially G1 phase, might contribute to the balance between proliferation and differentiation (Calegari et al., 2003; Calegari et al. 2005; Wilcock et al., 2007). We measured the length of cell cycle in E11.5 neuronal progenitors by cumulative BrdU-labeling during 9 hours (III, Fig. S3). We estimated that to explain the 50% thinning of VZ in mutants, the cell cycle length should be 35% longer (III, see Methods for an explanation). However, we could observe only a 3.2% longer cycle in $Fgfr1^{cko};Fgfr2^{cko}$ neuronal progenitors. Furthermore, statistical analysis indicate that a difference of this magnitude cannot explain the loss of progenitors in *Fgfr* mutants.

In our previous study we had shown that *Cyclins* were downregulated in the dorsal midbrain. However, in the *Fgfr1^{cko};Fgfr2^{cko}* ventral midbrain, mRNA expression of *CyclinD1* showed only a slight downregulation, whereas *CyclinB1* and *D2* appeared unaltered (III, Fig. S4). Furthermore, the reduced amount of *CyclinD1* mRNA was still able to produce a rather normal level of CyclinD1 protein, enough to propel cell cycle forward.

Disturbances in the apico-basal polarity of neuronal progenitors results in abnormal cell cycle progression and ectopic mitoses (Cappello et al., 2006; Costa et al., 2008). A complex of proteins, including Par3, Par6, Cdc42 and aPKCλ maintains this polarity. Other apical structures, such as primary cilia, are also considered to maintain proliferative progenitors, likely via mediating Shh signaling (Dubreuil et al., 2007). To understand whether these structures or cell polarity contributed to the premature neurogenesis, we analyzed *Fgfr1^{cko};Fgfr2^{cko}* VZ for components of the polarity-maintaining complex and apical structures. However, in mutants the junctional complexes, primary cilia and midbodies, as well as cell-polarity regulators appeared normal (III, Figs. 4, S5). In addition, mitotic cells were located near the apical surface of the VZ in mutants, providing further evidence that the cell polarity in mutants was unaffected (III, Fig. S1).

When neuronal progenitors divide, the daughter cell which retains at least some of the apical membrane components, adherens junctions, and the basal process, remains as a proliferating progenitor (Kosodo et al., 2004; Konno et al., 2008). The daughter cell which is left without leaves the cell cycle and starts to differentiate. The angle of mitotic spindle regulates the angle of cell division, and is thus thought to regulate the distribution of cellular components. We investigated whether the loss of FGF signaling could tilt the angle of mitotic spindle in an abnormal position and consequently result in more asymmetric divisions. However, most cells also in mutants were dividing vertically – i.e in a nearly 90 degree angle towards the apical surface (III, Fig. 4F). Statistical similarity analysis revealed that there were no significant differences between the distribution of cell division angles between wild-type and mutant progenitors.

Taken together, these results indicate that the loss of FGFR1/2-mediated signaling does not have a major effect on cellular properties nor cell cycle progression in the neuronal progenitors.

4.3.3. FGF8 protein forms an A-P gradient in the basal lamina

As mentioned above, in order to remain proliferative the neuronal progenitors require a connection to the basal lamina (Konno et al., 2008). Via these connections, cells might receive yet unidentified signals to support their progenitor-characteristics. We speculated that one such signal might be FGF8. To investigate that, we analyzed the localization of FGF8 protein (specifically, FGF8b isoform) in the developing embryo. Verifying the specificity of the antibody, FGF8 protein colocalized with *Fgf8* mRNA in all expressing tissues, such as in the rostral rhombomere 1, branchial arches and limb bud, and was lost in *Fgfr1^{cko}* midbrain (III, Fig. 5, S6).

In the isthmic organizer cells which expressed *Fgf8* mRNA, we could detect FGF8 protein in the cytoplasm and in the apical side. However, the strongest signal was localized in the basal lamina where the protein formed a gradient, diminishing away from the boundary. The mRNA of FGF targets *Dusp6* and *Spry1* formed a similar

gradient, corresponding to the FGF8 localization. In the forebrain, which is another expression domain of *Fgf8*, a similar basal localization of FGF8 was observed (Fig. S6).

In addition, in the lateral midbrain, phosphorylated forms of FGF targets ERK1/2 were detected not only in the soma of neuronal progenitors, but also in their basal processes. These structures were not affected in *Fgfr1*^{cko}*;Fgfr2*^{cko} midbrain, but the progenitors retained their connection to basal lamina normally. Next, we tested *in vitro* if neuronal progenitors could benefit from a contact with an extracellular matrix molecule laminin. Indeed, laminin-coating of culture plates helped midbrain-derived progenitors to remain as proliferative progenitors, when they were depleted from FGF signaling (III, Fig. 6).

Taken together, we detected an A-P gradient of FGF8 protein, highly concentrated in the basal lamina. Furthermore, basal processes of neuronal progenitors, contacting the lamina, showed staining for active form of ERK1/2, indicating active FGF-signaling. These results, together with our in vitro observations, suggest that neuronal progenitors might receive FGF8 via their basal lamina -contacting processes.

4.3.4. The cell-division mode in *Fgfr1*^{cko}*;Fgfr2*^{cko} neuronal progenitors is biased towards symmetric neurogenic divisions

Two main division types previously described in VZ neuronal progenitors are symmetric divisions, which produce two proliferating progenitors, and asymmetric divisions, which produce one proliferating daughter cell whereas the other daughter either forms a more committed progenitor or cell exits the cell cycle and begins to differentiate (Götz and Huttner, 2005). In addition, basal progenitors in the forebrain are able to produce two differentiating daughter cells by a symmetric neurogenic division. This far, cell-division types occurring in the ventral midbrain VZ had not been analyzed.

The premature neurogenesis observed in *Fgfr1*^{cko}*;Fgfr2*^{cko} midbrain could be caused by increased asymmetric cell divisions – without major alterations to the cell division plane as shown above – or increased symmetric neurogenic divisions. To study these two options, we analyzed the cell-division mode *in vitro* by a pair-cell assay under two conditions, with or without FGF2 (we speculated that an excess amount of FGF2 could mimic the combination of isthmic FGFs in this experimental setting). Progenitors were dissociated from E9.5 wild-type midbrain, at the time when no pos tmitotic neurons were yet present, and cultured for 21 hours, during which the neurogenesis normally begins *in vivo*. The status of each cell was identified by HuC/D and Sox2 staining. In the presence of FGF2, neuronal progenitors divided in equal amounts by s ymmetric proliferative, asymmetric neurogenic, and symmetric neurogenic divisions (III, Fig. 6). However, the lack of FGF biased this balance, and now most of the progenitors were dividing by symmetric neurogenic divisions, producing two neurons in each division.

4.3.5. Summary

Together, these results provide further understanding to the connection between FGF signaling and neuronal progenitor maintenance. According to our model, neuronal progenitors might receive FGFs via their connections to the basal lamina, to maintain *Hes1* expression in the ventral midbrain. *Hes1* in turn represses the expression of proneural genes, such as *Ngn2*, *Dll1* and *Mash1*, thus maintaining the neuronal progenitors in a proliferative state. When the progenitors lose their source of FGFs, their

cell division mode switches from symmetric proliferative to symmetric neurogenic divisions (III, Fig. 6). The loss of FGF signaling does not affect the cell cycle length, cell division plane, or apico-basal polarity of neuronal progenitors, nor their connections to the basal lamina.

4.4. FGF-regulated patterning of the meso-diencephalic dopaminergic domain (IV)

We had previously shown that *Fgfr1^{cko};Fgfr2^{cko}* and *Fgfr1^{cko};Fgfr2^{cko};Fgfr3^{null}* mutants display a gradual loss of TH in the midbrain, as well as a defect in terminal differentiation of dopaminergic neurons. Here we wanted to investigate the meso-diencephalic dopaminergic domain in more detail, and to analyze how, and at which stages, FGF signaling operates in this region. Furthermore, we wanted to know what happens to dopaminergic neurons in *Fgfr* compound mutants – whether they die, or change their identity. In addition, we analyzed chimeric embryos, described already in Study III, to investigate whether FGF signaling affects the development of dopaminergic neurons cell-autonomously.

4.4.1. FGF signaling components are expressed in dopaminergic progenitors

We first wanted to investigate whether FGF signaling operates only at the level of progenitors, or also in the postmitotic neurons. For this, we analyzed the expression of *Fgf-receptors 1* and *2*, as well as known FGF targets, in the wild-type embryonic midbrain during the time when dopaminergic neurons begin to form. We discovered that the expression of FGF signaling components was restricted to the proliferative dopaminergic progenitors, being absent in postmitotic precursors (IV, Fig. 1). In fact, FGF targets were downregulated in the midbrain dopaminergic domain by E 11.5. Furthermore, although *Fgfrs* were detected throughout the midbrain and diencephalon, FGF targets were restricted to the midbrain side.

4.4.2. A novel A-P pattern in the meso-diencephalic dopaminergic domain, regulated by FGF signaling

To analyze meso-diencephalic dopaminergic domain in more detail, we analyzed sagittal sections of E12.5 control embryos (IV, Fig. 2). We mapped the boundary between diencephalon and midbrain using the neuromeric model by Puelles and Rubenstein (2003), in which the dorsal structure, posterior commissure, determines the boundary position. The midbrain-hindbrain boundary was detected by Otx2 IHC on parallel slides. In order to label the midbrain and rhombomere 1 region boundaries, we used En1-Cre-mediated recombination of *R26R*. Unexpectedly, the recombined domain extended to the caudal diencephalon (IV, Fig. 2). In the dorsal side, the anterior border

of the recombined domain corresponded with the posterior commissure, i.e the meso-diencephalic boundary (data not shown).

As reported previously (Marín et al., 2005), we detected TH^+ cells not only in the midbrain, but also in the caudal diencephalon in E12.5 embryos (IV, Fig. 2). Some TH^+ cells were detected also anterior to the betagalactosidase$^+$ domain. We also discovered a novel A-P pattern in the dopaminergic domain. In the caudal diencephalon, dopaminergic neurons were intermingled with $FoxP1^+Pou4f1^+$ non-dopaminergic neurons (IV, Fig. 2 and Supplementary Fig. S1). In the midbrain side, dopaminergic neurons and $Pou4f1^+$ neurons were separated in different domains, m7 and m6, supporting previous observations (e.g. Agarwala and Ragsdale, 2002; Prakash et al., 2009; Kala et al., 2009).

As isthmic FGF signaling affects antero-posterior patterning in the midbrain and hindbrain region, we hypothesized that it likely similarly operates in the dopaminergic domain. Thus, we analyzed this domain in *Fgfr1^{cko};Fgfr2^{cko}* mutants. We discovered that in the mutant midbrain, the midbrain dopaminergic domain contained $FoxP1^+Pou4f1^+$ cells among TH^+ cells (IV, Fig. 2). However, D-V domains in the mutant ventral midbrain were unaltered, and $Nkx6.1^+$ cells were not found in the mutant dopaminergic region. This suggested that the excess $Pou4f1^+$ cells were not originating from the neighboring midbrain m6 domain.

Next, we investigated molecular differences between diencephalic and midbrain dopaminergic precursors in more detail. Diencephalic precursors lacked Pitx3, *DAT*, and *En2* (IV, Fig 3). The level of TH, as detected using IHC, appeared weaker in the diencephalon. In contrast, *Nurr1* and *Lmx1b* expression did not differ between diencephalon and midbrain. Compared to the midbrain, diencephalic dopaminergic precursors expressed less *Ddc*, and *En1* very weakly. In addition, *En1* and *En2* in the diencephalic VZ were absent, in contrast to midbrain where they were still weakly expressed at E12. In fact, *En1* was lost in the diencephalon already by E 9.5 (IV, Supplementary Fig. S2).

In mutants, dopaminergic precursors adopted a fate which greatly resembled that of diencephalic dopaminergic progenitors (IV, Fig. 3). This included the loss of *En1/2*, *DAT*, and Pitx3, and decreased expression of *Ddc*.

4.4.3. The loss of *En1* and *En2* in *Fgfr1^{cko};Fgfr2^{cko}* postmitotic dopaminergic precursors does not lead to apoptosis

As En1/2 are essential for the differentiation and survival of midbrain dopaminergic neurons (Simon et al., 2001), we investigated their expression in the control and mutant midbrain in more detail. Both *En1* and *En2* were detected in postmitotic precursors in dopaminergic domain, but also in more lateral midbrain, at E11.5 (IV, Fig. 4). In these lateral regions, both transcripts were detected in domains which produce glutamatergic red nucleus precursors, and GABAergic neurons (IV, Supplementary Fig. S3). Both *En1* and *En2* were still detected in proliferative dopaminergic progenitors in E10.5 mutants (IV, Supplementary Fig. S2). However, *En1* and *En2* were absent in the postmitotic dopaminergic precursors at E11.5, and one day later, both transcripts were lost also in more lateral regions (IV, Fig. 4).

Similarly to *Fgfr1^{cko};Fgfr2^{cko}* embryos, in *En1$^{-/-}$;En2$^{-/-}$* mutants the dopaminergic neuron development and TH expression begins normally. Shortly thereafter, however, *En-*

deprived dopaminergic precursors die by apoptosis (Simon et al., 2001, Alavian et al., 2009). In contrast to the situation in *En1*$^{-/-}$*;En2*$^{-/-}$ embryos, the *Fgfr1*^{cko}*;Fgfr2*^{cko} dopaminergic precursors did not display apoptosis, as detected by TH and active Caspase3 (Cas3) IHC (IV, Fig. 5 and data not shown). Supporting these observations, we detected postmitotic Lmx1a^{+} cells in E15.5 mutant midbrain, although they lacked TH at this stage.

4.4.4. Diencephalic and midbrain dopaminergic domains show differences already at the progenitor stage

Although we could see cl ear differences in the postmitotic precursors between diencephalon and midbrain, it is possible that they represent only transient characteristics. Because suitable tools for fate-mapping these cells were unavailable, we focused instead on the earlier development of these two populations. We speculated that if indeed these populations differed from each other, they might show differences already at the progenitor stage. We had already detected absence of *En1* and *En2* in the diencephalic domain from E9.5 onwards, and downregulation of *Aldh1a1* by E10.5 (IV, Fig. 3 and Supplementary Fig. S2). Next, we analyzed various *Wnts*, as well as *Shh* and *FoxA2*. In control embryos, *Shh*, *FoxA2*, *Wnt5a*, and *Wnt7b* were expressed in both diencephalic and midbrain dopaminergic progenitors (IV, Fig. 6, Supplementary Fig. S5). In mutants, these genes were expressed in the midbrain VZ. In addition, *Wnt5a* was detected in the residual TH^{+} precursors in mutants (IV, Supplementary Fig. S5). In contrast, *Wnt1*, *Wnt8b*, and *Aldh1a1* were absent at E12.5 in the control diencephalic VZ. In fact, *Wnt8b* was not detected in this region at any of the stages analyzed (IV, Supplementary Fig. S4). In mutants, these genes were not expressed in the midbrain (IV, Fig. 6 a nd Supplementary Fig. S4). Similarly, Wnt-target *Drapc1* displayed a gradient-like expression pattern, being more weakly expressed in the diencephalon. In mutants, the expression level in the midbrain resembled highly the expression in the diencephalon (IV, Fig. 6 a nd Supplementary Fig. S5). Taken together, these results support our hypothesis that in *Fgfr1*^{cko}*;Fgfr2*^{cko} embryos, the midbrain dopaminergic domain loses its typical molecular characteristics and instead adopts those of the diencephalic domain.

4.4.5. FGF signaling functions cell-autonomously in midbrain patterning

FGF8 has been shown to induce dopaminergic neurons in explants culture system, but the exact mechanism is still unclear (Ye et al., 1998). FGF signaling might be required in the generation of dopaminergic-permissive environment in the ventral midbrain, thus working non-cell autonomously. Our previous work (Study III) had demonstrated that FGFR1/2-mediated signaling maintained neuronal progenitors in the midbrain cell-autonomously. Now we hypothesized that FGFs might function in a similar manner in patterning, focusing our attention to the dopaminergic domain. We analyzed the expression of dopaminergic neuron -specific genes, as well as localization of Pou4f1^{+} cells, in chimeric embryos, which contained both wild-type and *Fgfr1*^{cko}*;Fgfr2*^{cko} mutant cells (IV, Fig. 7). Betagalactosidase, expressed from En1-Cre-recombined *R26R* locus, marked the mutant cells. Consistent with *Fgfr1*^{cko}*;Fgfr2*^{cko} phenotype, some TH^{+} cells were able to develop in the mutant region, but they resembled diencephalic dopaminergic precursors: they lacked Pitx3 and *En1*. Furthermore, proliferative progenitors in the mutant regions did not express *Wnt8b*, and they were Aldh1a1 negative. Similarly what we had detected in *Fgfr1*^{cko}*;Fgfr2*^{cko} embryos, Pou4f1^{+} cells

appeared in mutant Lmx1a^{+} region in chimeras, but were absent in the corresponding wild-type area. These results indicate that FGF-signaling patterns A-P dopaminergic domain cell-autonomously.

Furthermore, general morphology of the midbrain in these chimeric embryos appeared abnormal. VZ structure was uneven, and MZ even contained enclosed clusters of the VZ (not included in the manuscript; see **Figure 15**). However, the border between VZ and MZ (in the apico-basal direction) was maintained, suggesting that the polarity of individual neuronal progenitors remained intact. These data highly resemble the observations by Li and colleagues (Sunmonu et al., 2011a), obtained by electroporating a construct encoding a constitutively active FGFR1 into chick midbrain. It appears that in our chimeric mutants, disruption of FGF signaling similarly lead to cell-adhesion defects in the midbrain. The disruption was more pronounced in the caudal parts of the midbrain, indicating a presence of an A-P adhesion gradient.

4.4.6. Ectopic retinoic acid does not fully rescue Pitx3 in FGF-deficient dopaminergic neurons

Aldh1a1, which appears to be one of Pitx3 targets (Chung et al., 2005a; Jacobs et al., 2007) catalyzes the oxidation of retinol into retinoic acid. This gene is initially expressed throughout the midbrain and caudal diencephalon in the dopaminergic domain. Later it becomes restricted to a subset of neurons in the midbrain, and appears to be absent in diencephalic dopaminergic domain. Retinoic acid -supplemented diet rescued dopaminergic neurons in *Pitx3*^{null} embryos (Jacobs et al., 2007).

We had previously shown that both *Pitx3* and *Aldh1a1* were downregulated in *Fgfr1*^{cko}*;Fgfr2*^{cko} midbrain by E 11.5 (Study II). We attempted therefore to rescue dopaminergic neurons in mutants by supplementing retinoic acid in food to pregnant females, from the time when embryos were E9.5 to the time when they were E13.5. Increasing retinoic acid dose on mutant embryos was able to rescue only a small caudal subset of dopaminergic neurons, demonstrated by emergence of Pitx3^{+}TH^{+} cells. In the control embryos, retinoic acid increased the number of TH^{+}Pitx3^{+} neurons throughout the midbrain, probably by stimulating the progenitor proliferation (IV, Supplementary Fig. S6). This suggests that the transformed "diencephalic" precursors in mutants, which downregulate *Aldh1a1* by E12.5 even in the wild-type, are unable to respond to retinoic acid. Alternatively, the retinoic acid dose was too low to have a full impact, but on the other hand, increasing its amount only resulted in high embryonic lethality. However, our results also indicate that in *Fgfr1*^{cko}*;Fgfr2*^{cko} embryos, few midbrain dopaminergic progenitors still exist in the most caudal midbrain, and they are able to respond to retinoic acid. In addition, our results suggest that Aldh1a1 might promote acquisition of correct midbrain dopaminergic phenotype by inducing, directly or indirectly, *Pitx3*.

4.4.7. Inactivation of *Fgfr1* and *Fgfr2* in postmitotic dopaminergic neurons does not affect their function of survival

Although we could not detect expression of either *Fgfrs* or FGF signaling targets in postmitotic neurons, we could not rule out that FGF signaling might still affect also postmitotic neurons. Therefore, we inactivated *Fgfr1* and *Fgfr2* using DAT-Cre and Th-

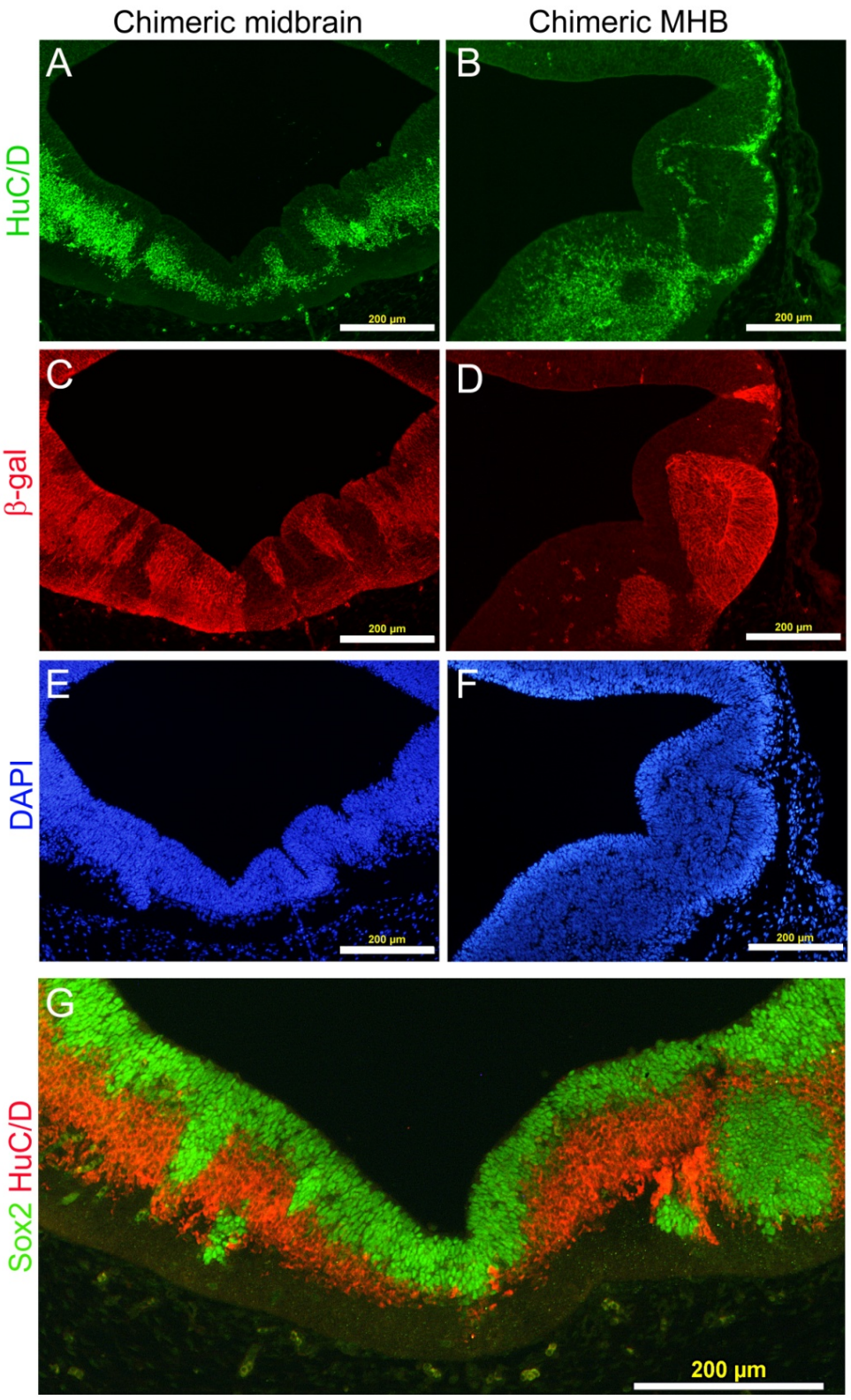

Figure 15. Abnormal structure in the chimeric *Fgfr1*^{cko};*Fgfr2*^{cko} mutant midbrain. (**A-G**) Coronal sections of E12.5 chimeric brain tissue. Midbrain in (A, C, E, G), and midbrain-hindbrain boundary region in (B, D, F). Beta-galactosidase IHC marks the mutant cell clusters for (A-F); not shown for (G). In many places, the VZ was bent inwards forming deep pockets within the neuroepithelium. Sometimes these VZ "pockets" even formed hollow tubes within the MZ (not shown). MHB, midbrain-hindbrain boundary region.

Cre, both of which begin to function in postmitotic dopaminergic precursors. Although DAT-Cre efficiently recombined R26R at E15.5 (data not shown), dopaminergic neurons developed normally in *DAT-Cre;Fgfr1^{cko};Fgfr2^{cko}* mutants (IV, Supplementary Fig. S7). No changes in the appearance of dopaminergic nuclei were detected in adult mutants either, and the level of dopamine in the mutant striatum was not significantly altered, either. Similar results were obtained from E18.5 *Th-Cre;Fgfr1^{cko};Fgfr2^{cko}* brains. Mutant animals in both strains were viable, and did not display any visible symptoms or abnormal behavior. However, systematic behavioral testing might reveal more subtle differences in these animals.

4.4.8. Summary

Taken together, these results suggest that in the A-P direction, at least two dopaminergic precursor populations are established in the developing midbrain and caudal diencephalon (IV, Fig. 8). FGF signaling establishes the correct A-P pattern cell-autonomously in dopaminergic progenitors. The midbrain dopaminergic progenitor population requires FGF signaling for their specification, whereas diencephalic population appears to develop independently of FGF signaling. The diencephalic population, in which TH$^+$ cells intermingle with Pou4f1$^+$ cells, can be distinguished from the midbrain dopaminergic domain by differential gene expression. For example, whereas midbrain population expresses strongly *En1* and *En2*, these genes are expressed either very weakly or not at all in the diencephalic precursors. In addition, at E12.5 diencephalic TH$^+$ neurons lack *Pitx3* and *DAT*, two genes which are characteristics of terminally differentiated midbrain dopaminergic neurons. In the absence of FGF signaling, midbrain dopaminergic precursors adopt an identity which resembles that of the diencephalic precursors.

5. DISCUSSION

5.1. Cooperation of FGFRs in midbrain-hindbrain development (I, II)

5.1.1. Expression of *Fgfrs* in the midbrain-hindbrain region

During mouse embryogenesis, *Fgfr1* is widely expressed throughout the embryo, including midbrain and hindbrain region. *Fgfr2* and *Fgfr3* are also expressed but in a more restricted pattern during E8.5 – E12.5 (Trokovic et al., 2003; Blak et al., 2005).

According to Trokovic et al., (2005), *Fgfr2* and *Fgfr3* are expressed in E9.5 midbrain and rhombomere 1, but are absent from the midbrain-hindbrain boundary. In contrast, Wurst and colleagues (Blak et al., 2005) show that *Fgfr2* is expressed weakly in the midbrain-hindbrain boundary already at E9.5, in the area corresponding to *Fgf8* expression gap. Supporting both of those results, in our Study IV, we show that at E10.5, *Fgfr2* expression is undetectable in the ventral midbrain side close to the boundary area, but in rhombomere 1 *Fgfr2* expression extends to the boundary region. From E11.5 onwards, we detect *Fgfr2* expression throughout the ventral midbrain, but it was still absent from the ventrolateral midbrain. *Fgfr3* expression was detected close to the boundary in rhombomere 1 side already at E9.5, and the expression extended towards the boundary region on both sides by E12.5 (Blak et al., 2005). Taken together, these data show that both *Fgfr2* and *Fgfr3* are weakly expressed at the midbrain-hindbrain boundary and they can participate in receiving FGF signals from the isthmic organizer. The expression of *Fgfr1* and *Fgfr2* in $Fgfr1^{cko}$, and *Fgfr3* in $Fgfr1^{cko};Fgfr2^{cko}$ mutants probably explains the residual expression of FGF targets in the ventral midbrain-hindbrain region.

Because the expression of *Fgfr2* and *Fgfr3* is absent in the areas where strongest FGF target expression was seen, it is possible that high levels of FGFR1-mediated signaling could repress the expression of other receptors. Indeed, it has been reported that *Fgfr3* is upregulated in zebrafish *ace* mutants, and *Fgf8b* is able to repress *Fgfr2/3* expression, but not *Fgfr1*, in embryonic mouse brain explants (Sleptsova-Friedrich et al., 2001; Liu et al., 2003). Consequently, in $Fgfr1^{cko}$ mutants both *Fgfr2* and *Fgfr3* might be upregulated near the boundary, and account for the residual target expression. However, *Fgfr2* appeared not to be upregulated at E9.5 in $Fgfr1^{cko}$ midbrain or hindbrain, although the whole mount ISH used in this study may not be sensitive enough to detect subtle differences (Trokovic et al., 2005). But even a much more sensitive method, microarray analysis of E10.5 $Fgfr1^{cko}$ (Study I) and E12.5 $Fgfr1^{cko};Fgfr2^{cko}$ midbrain and hindbrain (P.Peltopuro and J.Partanen, unpublished results) did not reveal *Fgfr2* nor *Fgfr3* among the upregulated genes. Thus, although we cannot fully exclude the upregulation of other FGF receptors in $Fgfr1^{cko}$ and $Fgfr1^{cko};Fgfr2^{cko}$ mutants, the receptor expression detectable already in the wild-type embryos, may account for the residual target expression in these mutants.

5.1.2. FGFRs 1,2, and 3 respond to isthmic signals cooperatively

The inactivation of *Fgfr2* and *Fgfr3* by itself does not result in a brain phenotype (Blak et al., 2005). This suggested that FGFR1 is the main receptor receving signals from the isthmic organizer. However, based on their expression patterns, we assumed that

FGFR2 and FGFR3 may be involved in the signal transduction in the area. This is supported by the fact that although the same En1-Cre was used to inactivate *Fgfr1* and *Fgf8*, the phenotype of *Fgfr1*^{cko} mutants was much less severe than that of *Fgf8*^{cko} embryos (Chi et al., 2003). In *Fgf8*^{cko} embryos, apoptosis occurring in the midbrain and rhombomere 1 between E8.5 and E10.0 results in the loss of entire dorsal midbrain, isthmus and cerebellum. In addition, the ventral midbrain structures, such as dopaminergic neurons and locus coeruleus, were lost in these mutants.

Indeed, the inactivation of *Fgfr1*, *Fgfr2*, and *Fgfr3* produced an increasingly more severe midbrain-hindbrain phenotype, demonstrating that these receptors do cooperately receive isthmic FGFs. *Fgfr1*^{cko} mice were viable and showed the mildest phenotype. Already *Fgfr1*^{cko}*;Fgfr2*^{cko} showed a much more drastic loss of midbrain-hindbrain tissue, and the phenotype of *Fgfr1*^{cko}*;Fgfr2*^{cko}*;Fgfr3*^{null} embryos was identical to *Fgf8*^{cko} embryos. The redundancy of receptors was further demonstrated by the downregulation of FGF target genes, which occurred earliest in *Fgfr1*^{cko}*;Fgfr2*^{cko}*;Fgfr3*^{null} mutants.

In conclusion, FGFR1 appears to be the most important FGFR operating in the midbrain-hindbrain region, and it is able to compensate for the loss of other two. *Fgfr1*^{cko}*;Fgfr3*^{null} embryos resemble *Fgfr1*^{cko} mutants, demonstrating that FGFR2 is likely able to compensate for the loss of FGFR3.

5.2. FGF signaling maintains neuronal progenitor survival and proliferation (II, III)

During embryogenesis, programmed cell death is required for sculpting tissues, deletion of transient structures, regulating cell number, and removing abnormal cells (Fuchs and Steller, 2011). Cells usually die via apoptotic pathway, which involves the activity of caspases, but nonapoptotic programmed cell death also exists – termed autophagic cell death and "necroptosis" (Yuan and Kroemer, 2010). To what extent these nonapoptotic pathways contribute to normal development is still unclear (Fuchs and Steller, 2011).

The role of FGFs as mitogens and survival-promoting factors has been widely reported. In midbrain-hindbrain-specific *Fgf8*^{cko} embryos, apoptosis occurs widely in dorsal midbrain-hindbrain regions during early embryogenesis (Chi et al., 2003). In *Fgfr1*^{cko} embryos, despite that these mice lack inferior colliculus and the vermis of cerebellum, no apoptosis was detected by TUNEL or Nissl Blue staining either at E9.5 or at E10.5 (Trokovic et al., 2003). FGFR2 and FGFR3 are likely able to maintain normal cell survival in these embryos. In addition, conditional inactivation of *Fgfr1-3* in the developing telencephalon leads to apoptosis of the anterior forebrain (Paek et al., 2009). Recently it was shown that FGF-signaling, promoted by Wnt-beta-catenin pathway, promotes cell survival via maintaining Myc (Paek et al., 2011). Myc in turn antagonizes proapoptotic TGF-beta signaling by repressing *p21* expression.

Supporting these results, we also detected extensive apoptosis in *Fgfr* compound mutants, mainly in the dorsal midbrain and rhombomere 1. In contrast, we detected very little apoptosis in the ventral regions. Similarly, loss of *Cyclins* was most prominent in the dorsal mid-hindbrain boundary, whereas ventrally, residual expression was seen. In ventral regions, cell cycle progressed normally despite somewhat decreased *Cyclin* mRNA expression. Interestingly, CyclinD1 protein levels in mutants were not significantly affected, suggesting that the residual mRNA is able to produce the required amount of protein.

The loss of *Cyclins* in dorsal regions could result from several factors. *Fgf17* and *Fgf18,* and *Fgf8a*, regulate cell proliferation in the midbrain and hindbrain, and these genes were downregulated in *Fgfr* compound mutants (Lee et al., 1997; Liu et al., 1999; Sato et al., 2001; Xu et al., 2000; Liu et al., 2003). In addition, isthmic *Wnt1*, known to affect cell proliferation in the midbrain (Panhuysen et al., 2004), as well as prevent apoptosis (Chen et al., 2001), was downregulated in *Fgfr* compound mutants, as was the canonical Wnt target *Drapc1* (Takahashi et al., 2002) in the dorsal midbrain. This downregulation pattern of *Drapc1* corresponded to the downregulation of *CyclinD2,* and to the region where greatest apoptosis was seen. It is likely that proliferation defect and apoptosis are connected – i.e when progenitors are unable to complete their cell cycle, they undego programmed cell death.

Rostral serotonergic neurons were especially vulnerable to the loss of FGF-signaling, as they disappeared already in $Fgfr1^{cko}$ mutants. However, we do not know whether these neurons began to develop and then died at a very early stage, or whether they failed to be induced.

5.3. FGF-signaling in the A-P patterning of the midbrain-hindbrain region (I, II, IV)

The interaction between *Otx2* and *Gbx2* has been shown to determine the position of midbrain-hindbrain boundary. Ectopic expression of *Otx2* in rhombomere 1 transforms this region to adopt midbrain identity, whereas ectopic *Gbx2* expands rhombomere 1 (reviewed in Joyner et al., 2000).

The partial downregulation of *Gbx2* likely explain the concomitant caudal shift in *Otx2* expression in $Fgfr1^{cko}$ mutants. In *Fgfr* compound mutants, the *Otx2* shift is even more prominent, likely due to increased apoptosis and reduced proliferation in dorsal tissues in these mutants. The loss of *Gbx2* domain in dorsal rhombomere 1 may result from the loss of isthmic organizer function but also from apoptosis, mentioned above, and decreased cell proliferation, demonstrated by dow nregulation of *Cyclins* especially in the dorsal midbrain and rhombomere 1 at E9.5.

The loss of dorsal tissue by a poptosis, and decreased cell proliferation, may both contribute to the caudal expansion of dorsal diencephalon. Although this is not clear at E9.5, already at E11.5 the wild-type tissue from diencephalon has expanded to the dorsal midbrain, replacing the dead tissue. By birth, this expansion is demonstrated by a larger posterior commissure especially in *Fgfr* $Fgfr1^{cko};Fgfr2^{cko};Fgfr3^{null}$ mutants, similarly what is observed in the $Fgf8^{cko}$ brain (Chi et al., 2003). It has been suggested that isthmic signals could regulate the position of rhombomere 1 – rhombomere 2 boundary by repressing *HoxA2* (Irving and Mason, 2000). However, the loss of rhombomere 1 tissue by apoptosis may also explain the apparent anterior expansion of *HoxA2* expression in $Fgfr1^{cko};Fgfr2^{cko}$ embryos.

R26R-reporter analyses at E12.5 showed that in $Fgfr1^{cko};Fgfr2^{cko}$ mutants, the anterior boundary of *En1* expression domain appeared unaltered. Based on the position of posterior commissure and ZLI in the wild-type embryos, this anterior border localizes in

the caudal diencephalon (Puelles and Rubenstein, 2003). As shown by electroporation experiments in chick, the tectal meso-diencephalic boundary is regulated by mutual repression between *En1* and *Pax6* (e.g. Mastick et al., 1997; Araki and Nakamura, 1999). Generally it has been believed that *En1* expression might determine the whole midbrain and anterior hindbrain region. However, the electroporation experiments in these studies were done only on the tectal region, and it is unclear whether similar interaction occurs in the ventral part. Indeed, we found En1-Cre-recombined tissue in the ventral prosomere 1, possibly also in prosomere 2. Defining the exact anterior limit of *En1* expression requires more detailed analysis of prosomere boundaries.

5.3.1. Red nucleus

We found Pou4f1$^+$ expressing cells in the anterior Lmx1a$^+$ domain, where they were intermingled with TH$^+$ neurons (see below). According to Puelles (1995), parvocellular red nucleus develops in prosomere 1. Thus, the Pou4f1$^+$ cells we observed could contribute to the prospective parvocellular part. In addition to *Pou4f1*, the developing midbrain red nucleus expresses also *Nkx6.1* and *Emx2* (Agarwala and Ragsdale, 2002; Prakash et al., 2006). Whereas *Emx2* was not analyzed in our studies, *Nkx6.1* was not expressed in these Lmx1a$^+$Pou4f1$^+$ cells. Indeed, rostral Pou4f1$^+$ neurons were able to develop in *Nkx6.1^{cko}* embryos (Prakash et al., 2009). Another transcription factor gene, which appears to be expressed in early midbrain but not in diencephalic red nucleus is *Pou2f2* (our own unpublished data). This gene was among the upregulated genes in *Fgfr1^{cko}* mutants, but it is not upregulated in Lmx1a$^+$ region of *Fgfr1^{cko};Fgfr2^{cko}* midbrain. The observed differences in the cellular composition and projections of magno- and parvocellular parts of adult red nucleus might originate from these early differences in transcription factor codes.

FGF signaling represses the size of both of these Pou4f1$^+$ domains. The effect is clearer in Lmx1a$^+$ region, but also the red nucleus in midbrain m6 domain spreads caudally. Given that medio-lateral patterning remains unaffected in *Fgfr* mutants, the excess Pou4f1$^+$ neurons do not migrate from m6 to m7 domain. Similar phenomenon – Pou4f1$^+$ cells in m7 – has been observed in *Lmx1b* mutants (Deng et al., 2011). In these embryos, isthmic organizer, and thus FGF-signaling, is disrupted. Thus, the phenotype in these mutants likely results from similar A-P patterning defects seen in *Fgfr* compound mutants, but this possibility was not addressed by the authors.

5.3.2. Motoneurons, locus coeruleus and serotonergic neurons in FGFR signaling mutants

Ventrally located III and IV cranial ganglia are lost in compound *Fgfr* mutants and in *Fgf8^{cko}* embryos (Chi et al., 2003). Based on neurofilament stainings and *Phoxa2* expression, these structures either are not induced, or are lost in a very early stage. According to *in vitro* experiments (Ye et al., 1998), Islet1$^+$ motoneurons do not appear to be very sensitive to the loss of FGFs. Thus, their disappearance in *Fgfr* compound mutants might not be a direct result from the loss of FGFs, but rather stem from the A-P patterning defect, which destroys a suitable "niche" for these ganglia. Alternatively, reduced proliferation or increased apoptosis might explain the loss of these nuclei.

Similarly, locus coeruleus, which is still present in *Fgfr1^{cko}* mice, is lost in *Fgfr* compound mutants. As this nucleus originates in dorsal rhombomere 1 where most prominent cellular death is detected, apoptosis may explain the loss of this cell

population. Cells in locus coeruleus appear more scattered in *Fgfr1*^{cko} mutants compared to the wild-type. This resembles the phenotype of *En1*$^{-/-}$;*En2*$^{+/-}$ and *En1*$^{-/-}$;*En2*$^{-/-}$ mutants (Simon et al., 2005). In these *En* mutants, locus coeruleus is lost and nucleus subcoeruleus, whose appearance is less compact, is preserved underneath. It is possible that the nucleus remaining in *Fgfr1*^{cko} embryos is in fact nucleus subcoeruleus. Furthermore, as isthmic organizer is disrupted in *En1/2* compound mutants, the described phenotypes result from decreased FGF-signaling in the region, rather than from the loss of *En1* and *En2* directly.

Rostral serotonergic neurons, which later will form dorsal raphe nucleus, develop in ventral rhombomere 1 in close vicinity to the FGF8 source (reviewed in Cordes 2005). These neurons likely receive a high dose of FGF8 during the early development, and are probably sensitive to the downregulation of this signal. Indeed, rostral serotonergic neurons are lost very early already in *Fgfr1*^{cko} embryos. Experiments with *in vitro* rat neural transplant system showed that FGF4 was required, together with Shh and FGF8, for the induction of rostral serotonergic neurons (Ye et al., 1998). Higher levels of other FGFs, including FGF8, were unable to produce the same effect. The authors concluded that although FGF4 is not expressed in the hindbrain at the time of serotonergic neuron induction, it could mimic the activity of earlier FGF4 inductive signal from primitive streak. In any case, the possible early FGF4 signal does not protect rostral serotonergic neurons later if the isthmic source of FGFs is compromised.

En transcription factors are needed in a dose-dependent manner for the development of serotonergic neurons (Simon et al., 2004). This requirement is non-cell-autonomous, because serotonergic progenitors do not appear to express either *En1* or *En2*. In *Fgfr1*^{cko} mutants, however, *En1* and *En2* expression is reasonably well preserved in the ventral midbrain and rhombomere 1, although it is lost dorsally. Similarly to the situation with locus coeruleus and *En* genes (see above), the serotonergic phenotype in *En1/2* compound mutants might stem from the downregulation of *Fgf8* in the isthmic organizer.

5.4. FGFRs affect cell-autonomously A-P patterning of dopaminergic domains (I, II, IV)

5.4.1. Expression of *Fgfr1* and *Fgfr2* in dopaminergic progenitors

At E10.5, when the majority of dopaminergic progenitors are still proliferating, both *Fgfr1* and *Fgfr2*, and their targets *Erm*, *Pea3* and *Dusp6* were expressed in *Aldh1a1*-expressing area of ventral midbrain. The targets appeared restricted to the caudal part of midbrain. Both receptors were clearly expressed in progenitors only. In addition, the expression of *Fgfr3* is similarly restricted to the VZ, and absent in the postmitotic dopaminergic cells (our own unpublished data, and Blak et al., 2005).

Although the expression of *Fgfrs* appears undetectable in mature neurons by ISH, this does not necessarily mean that they are completely absent. Indeed, several studies have reported that FGF signaling functions in mature midbrain dopaminergic neurons. Dominant negative FGFR under *Th*-promoter affected the function, but not majorly survival, of the midbrain dopaminergic neurons (Klejbor et al., 2006). In addition, FGFs

have been implicated in neurological disorders, and FGF2 level is increased in the peripheral blood of schizophrenic patients (Hashimoto et al., 2003). Thus, it is possible that FGFs are involved in the function of mature dopaminergic neurons to some extent. For example, if the receptors are accumulated in the synapses of mature dopaminergic neurons, a very low mRNA expression level can likely maintain an adequate amount of receptors. An indirect effect may also be possible: other *Th*-expressing, FGF-dependent neurons might normally provide support to midbrain dopaminergic neurons. Loss or malfunction of these supportive afferents might affect the function of their target neurons.

Alternatively, the more severe phenotype observed in the dominant negative *Fgfr* mutant mice might be due to a more effective blockage of FGF signaling by the dominant receptor, which is able to prevent signaling via all FGFRs. The lack of phenotype in *DAT-Cre* or *Th-Cre Fgfr* compound mice may thus result from compensation by wild-type *Fgfr3*, possibly also *Fgfr4*.

Despite the observed efficient *R26R* recombination, it is possible that the Cre-lines used in our study have not fully inactivated the *Fgfr* alleles, and thus the residual receptor expression could explain the normal phenotype. As mRNA levels of *Fgfr1* and *Fgfr2* are so low, verifying *Fgfr* and FGF target expression in these mutants would require more sensitive approaches, such as qPCR.

5.4.2. Different dopaminergic domains in midbrain and caudal diencephalon

We identified a novel antero-posterior pattern of dopaminergic progenitors and precursors in the midbrain and caudal diencephalon (**Figure 16**). At E12.5, the diencephalic domain is clearly identified by the presence of $Pou4f1^+$ non-dopaminergic precursors among TH^+ cells. Diencephalic dopaminergic progenitors and precursors appear to differ from their midbrain counterparts in their expression of several genes involved in dopaminergic neuron development and maturation, such as *En1*, *En2, Pitx3*, *Ddc*, and *DAT*.

It is possible that there is only a delay in the expression of these genes, and normally in the wild-type they would be expressed at some point. However, also proliferative dopaminergic progenitors in the diencephalon show different molecular characteristics compared to the midbrain. This includes lack of *Wnt8b*, and downregulation of *En1*, *En2*, *Aldh1a1*, and *Wnt1* already at early embryonic stages. These early differences suggest that dopaminergic progenitors in the diencephalon adopt a fate different from those in the midbrain, and may not even contribute to the mature meso-diencephalic dopaminergic system. Thus, proper dopaminergic neurons might only develop in the midbrain population, close to the isthmic FGF8 source. However, some $Pitx3^+$ neurons are observed in the lateral regions of anterior meso-diencephalic domain at E12.5, and they may later become SNpc neurons (Maxwell et al., 2005). Fate-mapping studies should clarify whether SNpc neurons are actually born in the midbrain, and then migrate to the anterior-lateral region.

TH^+ cells which lack other typical markers of dopaminergic neurons exist in different parts of the brain (reviewed in Björklund and Dunnett, 2007). It is possible that the diencephalic TH^+ neurons will later lose TH expression and adopt an alternative neurotransmitter identity. For example, some of the 'atypical' TH^+ neurons mentioned above have GABAergic morphology, and can even be $GABA^+$ and TH^+ (Björklund and

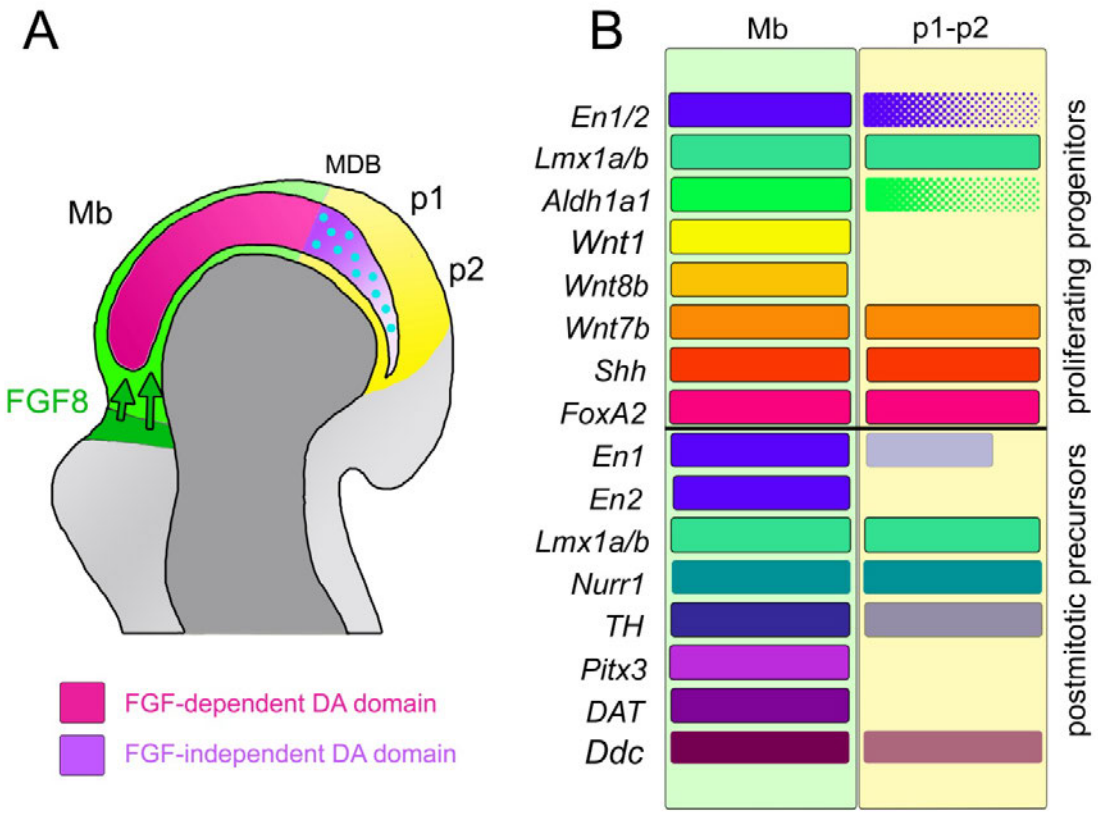

Figure 16. Different molecular profiles in the dopaminergic precursors in diencephalon and midbrain. **A**) Schematic sagittal view of E12.5 ventral midbrain (green) and caudal diencephalon (yellow). FGF8 from the isthmus promotes the development of midbrain dopaminergic neurons. Diencephalic domain appears to be less dependent on isthmic FGF. In diencephalon, dopaminergic precursors are intermingled with Pou4f1^{+} cells (blue dots). (**B**) Gene expression in the proliferating dopaminergic progenitors and postmitotic precursors in the midbrain (green) and diencephalon (yellow). *En1/2* and *Aldh1a1* are expressed early in progenitors, but lost by E12.5 (hatch pattern). *Wnt1* and *Wnt8b* are not expressed in the diencephalon side. *En1* is very weakly expressed in postmitotic diencephalic precursors (lighter blue), but absent in the most anterior precursors. *Th* and *Ddc* are more weakly expressed in the diencephalon, compared to the midbrain (lighter color). Diencephalic dopaminergic neurons entirely lack *Pitx3* and *DAT*, both characteristics of terminal differentiation in the midbrain.

Dunnett, 2007). Alternatively, these neurons could represent a transient structure, like the subplate, and only needed during early development. In any case, fate-mapping studies are needed to elucidate the fate of these neurons. This requires identification of a gene which is specifically expressed in diencepalic TH^+ progenitors but never in the midbrain population. If the observed differences between the diencephalic and midbrain dopaminergic neurons persist also later, it means that the early TH positivity cannot be used as a reliable marker of the fact that a neuron will adopt a fully functional dopaminergic phenotype. Other markers, and their expression dynamics, also need to be monitored.

5.4.3. FGF signaling in the differentiation of midbrain dopaminergic neurons

In *Fgfr* compound mutants, TH^+ cells began to develop and at E11.5, their amount in *Fgfr1*^{cko}*;Fgfr2*^{cko} embryos resembled that in the wild-type. However, one day later the number of TH^+ neurons was reduced, and by birth, all TH in the midbrain was lost. Furthermore, markers of terminally differentiated dopaminergic neurons, such as *Pitx3* and *DAT*, are not expressed in the mutant midbrain. We show that in *Fgfr1*^{cko}*;Fgfr2*^{cko} embryos, original midbrain dopaminergic precursors appeared to change their identity to diencephalic ones. This change is characterized by a loss of several genes which are normally expressed in midbrain, but not in diencephalic, dopaminergic progenitors and precursors. As *En1* and *En2* are important patterning genes in the midbrain-hindbrain region, their gradual downregulation in *Fgfr1*^{cko}*;Fgfr2*^{cko} embryos might directly lead to the observed A-P patterning defects. It would be of interest to investigate whether *En1/2* mutants display a similar A-P defect in the dopaminergic domain.

Furthermore, this effect of FGF signaling appears to be cell-autonomous, as demonstrated by analysis of chimeric mouse embryos. Wild-type cell clusters retained their correct midbrain identity, and were able to develop into fully maturated dopaminergic neurons. In contrast, the neighboring mutant clusters adopted a caudal diencephalic identity due to loss of FGF8-mediated patterning signals. In these mutant clusters, TH^+ precursors resembled the diencephalic ones.

Whether the eventual loss of TH expression in *Fgfr* compound mutants is due to a possible fate change which occurs also in wild-type diencephalic TH^+ neurons, or whether it is a defect separately regulated by FGF signaling, remains to be determined. The dopaminergic phenotype appears to be even more severe in *Fgfr1*^{cko}*;Fgfr2*^{cko}*;Fgfr3*^{null} embryos, in which only a small population of rostrally located TH^+ cells develop in the midbrain (Study II). Although isthmic *Fgf8* is mostly downregulated already in *Fgfr1*^{cko}*;Fgfr2*^{cko} mutants, residual signals may be enough to support dopaminergic progenitors via *Fgfr3*. Alternatively, the more anteriorly located progenitors might even respond to FGF8 diffusing from ventral prosomere 3, if the protein is allowed to diffuse across ZLI. The more drastic loss of TH^+ cells in *Fgfr1*^{cko}*;Fgfr2*^{cko}*;Fgfr3*^{null} embryos could also be due to increased apoptosis in the ventral regions in later stages than analyzed in Study II. Nevertheless, as by birth midbrain dopaminergic neurons are lost in both mutants, *Fgfr1*^{cko}*;Fgfr2*^{cko} embryos are a sufficient model system to study FGF signaling in dopaminergic neuron development.

From these data, we conclude that isthmic FGF signaling cell-autonomously participates in the A-P patterning of meso-diencephalic dopaminergic domains. Diencephalic TH^+ neurons appear to be less dependent on isthmic FGF signals than midbrain dopaminergic neurons. This corroborates data from zebrafish that diencephalic

dopaminergic neurons do not require isthmic FGF8 for their specification (Holzschuch et al., 2003). Without FGF signaling, midbrain-type dopaminergic neurons cannot acquire their correct molecular profile, and they cannot maintain their *Th* expression. However, this requirement of FGF-signaling is restricted to proliferative progenitors, as the inactivation of *Fgfr1* and *Fgfr2* in postmitotic dopaminergic neurons did not lead to similar defects. It is possible that FGF signaling operating in midbrain dopaminergic progenitors activates a genetic program which ensures that a normal differentiation continues also in the postmitotic – FGF-independent – stage. Whether dopaminergic precursors in *Fgfr* compound midbrain could be rescued by a forced expression of transcription factors such as *En1/2*, or *Pitx3*, remains to be investigated.

Wnt1 has been suggested to regulate terminal differentiation of midbrain dopaminergic neurons (Prakash et al., 2006). In $Wnt1^{-/-}$ neural tube explants, FGF8-bead was unable to induce TH^{+} dopaminergic precursors. In $Fgfr1^{cko};Fgfr2^{cko}$ embryos, *Wnt1* was initially expressed in the ventral midbrain but then gradually downregulated, and its loss might contribute to the observed dopaminergic phenotype. However, the lack of *Th* and *Pitx3* in $Wnt1^{-/-}$ explants may be a secondary defect due to a decreased ability to respond to FGF8. Given that *Wnt1* is expressed earlier than *Fgf8* in the midbrain-hindbrain boundary, it may generally promote FGF8-competence of this region – i.e induce FGF8-responsiveness in neuronal progenitors.

5.5. FGFs maintain neuronal progenitors in the midbrain-hindbrain region (I, II, III)

5.5.1. FGF signaling maintains *Hes1* and *Sox3*

Loss of FGF signaling leads to increased neurogenesis in the midbrain-hindbrain region, which was apparent already in $Fgfr1^{cko}$ mutants. It was marked by the appearance of postmitotic neurons in the boundary region, and expansion of neurogenic gene-expression gradients towards the boundary. In *Fgfr* compound mutants, the effect was more pronounced. Postmitotic neurons appeared in the midbrain of $Fgfr1^{cko};Fgfr2^{cko};Fgfr3^{null}$ embryos already at E9.5. The premature neurogenesis resulted in the depletion of neuronal progenitor pool, which, together with increased apoptosis in the dorsal regions, likely accounted for the smaller size of the dorsal brain regions in these embryos. However, not all neuronal progenitors were lost. Thus, other signals, such as Wnts, likely participate in the progenitor maintenance.

Neuronal progenitors are maintained by transcription factors belonging to Hes and SoxB1 families. They antagonize the function of proneural factors, such as Mash1 and Ngn2, which induce neuronal differentiation. In $Fgfr1^{cko}$ mutants, *Hes3* was downregulated in the midbrain-hindbrain boundary, which contributed to increased neurogenesis in this region. In *Fgfr* compound mutants, *Hes1* was additionally lost in the ventral midbrain (**Figure 17**). Concomitantly, proneural genes were upregulated. Another Notch-effector *Hes5* was not significantly downregulated. It appears that FGF signaling can directly activate *Hes1* without cross-talk with other Notch signaling components. Indeed, in the developing cortex, FGF-FRS2-ERK1/2 can induce *Hes1* (Sato et al., 2010). This also corroborates observations from early stages of neural development, during which *Hes1* and *Hes3* expression is independent of Notch-pathway

(Kageyama et al., 2007). Hence, the regulation of *Hes* genes in the developing nervous system appears to be tissue- and timepoint specific.

SoxB1 proteins Sox1-3 maintain neuronal progenitor identity (Pevny and Placzek, 2005). *Sox3*, but not *Sox2*, was clearly downregulated in *Fgfr* compound embryos and slightly downregulated in *Fgfr1*^{cko} embryos. In zebrafish, several *Sox* genes including *Sox3* have been shown to positively regulate *Hes* class transcription factors, such as *Her3* (Okuda et al., 2010), preventing neurogenesis. Thus, downregulation of *Sox3* could contribute to loss of *Hes1* in the ventral midbrain. Furthermore, Sox proteins participate in a partner- and tissue-specific manner in patterning and neural fate specification (Okuda et al., 2010; Lefebre et al., 2007). It is tempting to speculate that *Sox3* could participate in the specification of midbrain dopaminergic neuron progenitor identity. In *Sox3* hypomorph brains, however, TH^{+} neurons appeared to develop normally (P. Peltopuro and J. Partanen, unpublished results).

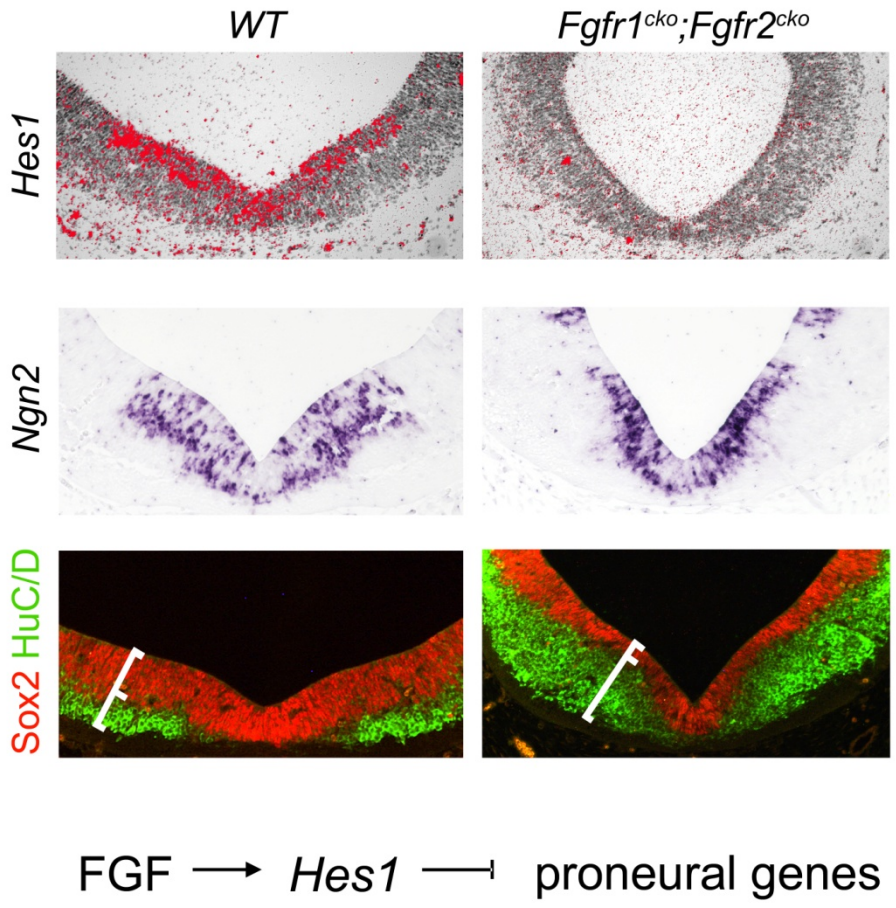

Figure 17. FGF-signaling maintains proliferative neuronal progenitors via *Hes1*.
A summary of results about neurogenesis in Study III. FGF signaling maintains *Hes1* expression in the ventral midbrain VZ. In *Fgfr* compound mutants, this expression is lost by E11.5, which results in the upregulation of several proneural genes, such as *Ngn2*. As a consequence, neurogenesis accelerates ,which depletes the Sox2^{+} progenitor layer and thickens HuC/D^{+} layer.

5.5.2. FGFs maintain symmetric proliferative divisions

Neuronal progenitors can divide in a symmetric or asymmetric manner. From asymmetric divisions, either a more committed progenitor type or a neuron or a glial cell is produced. Symmetric divisions in the VZ are thought to be proliferative, and symmetric neurogenic divisions have been described in basal progenitors in the forebrain (Götz and Huttner, 2005). By analyzing cell division types *in vitro*, using neuronal progenitors dissociated from ventral midbrain tissue before the onset of neurogenesis (E9.5), we were able to detect cell division types the previously described in the forebrain VZ: symmetric proliferative and asymmetric neurogenic ones. In addition, we showed for the first time that that some neuronal progenitors in the midbrain VZ are able to divide in a symmetric neurogenic manner. As this mode of cell division produces neurons very efficiently compared to an asymmetric division, it is logical that for a rapid onset of neurogenesis observed from E9 to E10, these types of divisions would be optimal. FGF signaling appears to be important for the maintenance of symmetric proliferative divisions, as in the absence of FGFs, most symmetric divisions produced two neurons instead of two proliferating progenitors.

It should be emphasized that our pair cell assay setup, as *in vitro* systems in general, can never recapitulate fully *in vivo* conditions, especially with neuronal progenitors which lose their polarity upon dissociation. Live cell imaging using tissue sections of midbrain and individual fluorescently labeled progenitors could shed more light into the division dynamics of midbrain neuronal progenitors, and the effect of FGFs on it.

5.5.3. FGF8 gradient in the basal lamina

The basal process has been suggested to be important for retaining neuronal progenitor identity (Konno et al., 2008). However it is unclear why a connection to the basal lamina is so important. One attractive hypothesis is that progenitors could receive "stemness-supporting signals" via their basal lamina contacts (Fishell and Kriegstein, 2003). We show that *in vivo*, FGF8b protein from the isthmic organizer forms a gradient in the basal lamina of neuroepithelium. Similar basal distribution was also seen in other places of neuroepithelium where *Fgf8* was expressed, such as in the ventral forebrain. In the expressing cells themselves, the protein was seen throughout the cells and even appeared apically accumulated, suggesting secretion to the apical side. In experiments where secretion of FGF8 from isthmus is chemically blocked, the protein accumulates under the apical surface of the expressing cells and signal in the basal lamina is lost (I. Crespo-Enríquez and D. Echevarría, personal communication). Thus it appears that FGF8 is secreted to the apical side and transported to basal lamina, where it diffuses to target tissues. In the study by Grove and colleagues (Toyoda et al., 2010), GFP-tagged FGF8 can be seen in the basal lamina of midbrain and strongly in the isthmus. Some apical staining can also be seen, although the authors do not provide close-up images of the midbrain region. Thus, some FGF8 may normally be distributed along the apical side, but that this signal is either lost during tissue processing, or too weak to be seen by IHC. However, FGFs interact with HSPGs, which are an essential component of the extracellular matrix in the basal lamina. Thus, the basal lamina would provide an ideal, stable platform for growth factor distribution. According to the current knowledge, a similar lamina does not exist in the apical side of the VZ, although various HSPGs can be found throughout the VZ (Ford-Perriss et al., 2003).

Integrins in the basal processes contact the basal lamina and might further amplify the contact between growth factors and their receptors (ffrench-Constant and Colognato,

2004). In our simple *in vitro* assay, the mere presence of laminin in the culture well helped the progenitors to remain proliferative. Although this assay cannot recapitulate the complex extracellular matrix, it indicates that basal lamina contacts might enhance progenitor maintenance, together with FGFs. Maybe FGF8-induced FGFR activation is synergistically promoted by l aminin-activated integrins. In a neural progenitor cell culture, beta-1 integrins activated MAPK, but not P13-K, pathway which was required for their maintenance (Campos et al., 2004).

In *perlecan*$^{-/-}$ mutants, in which basal lamina is disrupted, cell cycle progress in the forebrain is slowed down (Girós et al., 2007). The authors suggest that basal lamina - bound FGF2 could partly be required for the maintenance of neuronal progenitors, although they did not provide direct evidence for this. However, in a contrasting study, Götz and colleagues reported that progenitors which had lost their basal lamina contacts were able to proliferate and differentiate normally, only the laminar organization of the forebrain was significantly affected (Haubst et al., 2006). In these mutants, the size of forebrain was reduced at E14 and also VZ appeared thinner. It is possible that more alterations could be revealed in a more detailed analysis. Alternatively, as neuronal progenitors especially in the forebrain are heterogenous (Pinto and Götz, 2007), different progenitor types might display variable sensitivity to the loss of basal contacts. The remaining progenitors in these mutants could be OSVZ progenitors, which express many of the same molecular markers as VZ progenitors, such as *Pax6* and *Hes1* (Fietz et al., 2010; Hansen et al., 2010; Shitamukai et al., 2011).

Based on our *in vivo* observations, we propose that progenitors might receive FGF8 via their basal lamina contacts (**Figure 18**). Activated form of ERK1/2 detected in the basal processes also indicates active FGFR signaling at basal-lamina contact points. The basally derived FGFs then support the self-renewal status of progenitors by maintaining *Hes1*, which suppresses neurogenesis and thus promotes symmetrical proliferative divisions. When progenitors are depleted from FGFs, they lose *Hes1* which leads to upregulation of proneural genes. This in turn induces them to exit cell cycle. As basal lamina contacts cannot provide the necessary signal in mutants, also daughter cells which retain both apical and basal contacts might now exit the cell cycle, leading to more symmetric neurogenic divisions.

It is also possible that at least in the *Fgf8*-expressing cells, FGF8 could signal back to the cells in an autocrine fashion from the apical side. The polarity of the signaling could even result in the activation of different downstream pathways, if the localization of different FGF signaling targets is similarly apico-basally polarized.

However, we have not directly shown a functional connection between FGF8b isoform and the maintenance of *Hes1* expression. There is plenty of evindence that FGF8b is the molecule which is responsible for the patterning activity of the isthmic organizer. We could not detect FGF8b signal in the anterior part of midbrain, which may be also due to the weak sensitivity of the antibody. However, to act as a patterning molecule, the *absence* of FGF8b in the most anterior midbrain would similarly function as a patterning "signal". For *Hes1* expression and progenitor maintenance, cells in the most anterior midbrain would also need a FGF protein supply. Thus, other FGFs might be involved and FGF8b gradient might in fact mainly regulate patterning.

Several investigators have demonstrated that FGF8a, as w ell as FGF17 and FGF18, appear to lack patterning activity and be more involved in controlling the proliferation in the midbrain. It would be interesting to see whether these proteins are able to form similar gradients in the basal lamina, and whether these gradients extend further than

FGF8b. Similarly, it might be of interest to investigate whether the increased proliferation seen in the overexpression models are due to a more rapidly progressing cell cycle, or to an inhibited cell cycle exit. In other words, whether FGF8a/17/18 are also able to suppress neurogenesis, and whether they could directly counteract the pro-neurogenic function of FGF15.

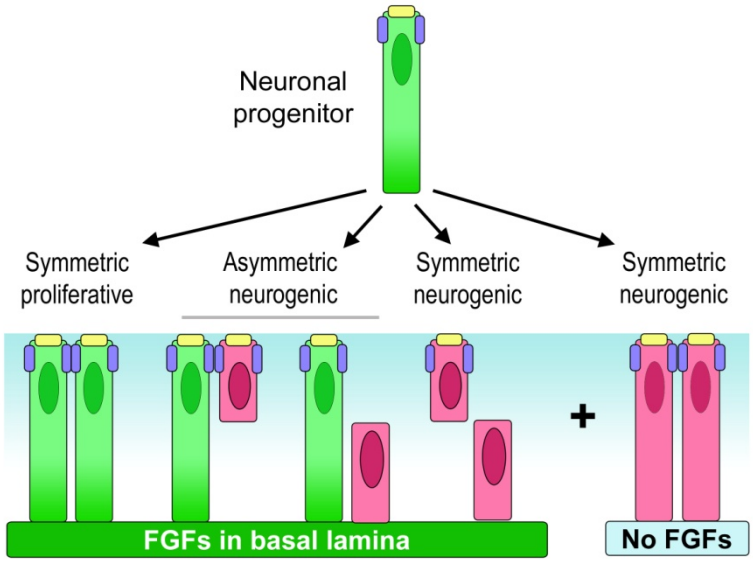

Figure 18. Basally derived FGFs maintain symmetric proliferative divisions.
A model of FGF-regulated division types in the midbrain VZ, based on Study III. See Figure 6 for an explanation of the cell division types. FGFs, secreted from the isthmic organizer, form an A-P gradient in the extracellular matrix of the basal lamina. In the wild-type VZ, neuronal progenitors receive FGFs from the basal lamina via their basal process. Progenitors which retain both apical components and the basal process – and thus a contact to the FGF source – remain as progenitors (green). When the progenitors cannot receive FGFs from the basal lamina, they begin to differentiate (red). In this scenario, inheriting the basal process does not prevent daughters from exiting the cell cycle, and symmetric proliferative divisions turn into symmetric neurogenic ones.

CONCLUDING REMARKS

Before the onset of this study, the importance of isthmic FGF8b as the major patterning molecule in the region was already well recognized, and the findings were supported by conditional *Fgf8* mouse mutants as well as zebrafish *acerebellar* mutants. In the previous studies in our group, Ras and Nina Trokovic had shown that FGFR1 is required for the correct development of midbrain and rhombomere 1. H owever, neurogenesis or the development of serotonergic or dopaminergic neurons in these mutants had not been analyzed. Gail Martin and colleagues had shown that conditional *Fgf8* mouse embryos lacked most of the dorsal midbrain and hindbrain, including cerebellum, as well as all dopaminergic neurons in the ventral midbrain. In contrast, our conditional *Fgfr1* mutant mice were viable although displayed defects in motor coordination. Furthermore, members of our group, and our collaborators in Germany (Wolfgang Wurst and co-workers), had demonstrated that the inactivation of *Fgfr2* and *Fgfr3* alone or as a combination does not affect the brain phenotype, suggesting that FGFR1 was the main receptor in this region. Taken together, these observations indicated functional redundancy between FGFRs.

In this study, we further analyzed the role of FGF signaling in the developing mouse midbrain and anterior hindbrain. We have demonstrated that all three FGF receptors (FGFR1-3), which are expressed in this region, cooperate to receive isthmic FGFs. The redundancy of FGF receptors was manifested by the expression of FGF target genes, cell survival, neurogenesis, patterning, and development of several neuronal populations, such as midbrain dopaminergic neurons. Furthermore, we showed that FGF signaling regulates both neurogenesis and patterning in this region directly.

We have shown that FGF signaling is required for cell survival and efficient proliferation, especially in the dorsal regions; and generally for the maintenance of proliferative neuronal progenitors via *Hes1,* and possibly via *Sox3*. We also demonstrated that FGF8b protein was highly concentrated in the basal lamina, where it formed a gradient. Thus, FGF8 could be a basal-lamina-derived signal which maintains proliferative neuronal progenitors.

In addition, different neuronal populations in the midbrain-hindbrain region responded differently to the loss of FGF-signaling. We showed that the rostral serotonergic neurons are especially sensitive to a decrease in FGF signaling. Their disappearance does not result from an A-P patterning defect, but appears to be independently regulated by the loss of FGFs. Whether serotonergic progenitors fail to be induced, are lost by apoptosis, or change fate, could be an interesting topic for future studies. Some FGF-regulated genes, discovered in our microarray analysis, showed strong upregulation specifically in the most anterior hindbrain. Investigating these genes more closely could reveal novel players in the development of this region. Other upregulated genes similarly showed interesting expression patterns in the VZ, MZ, or both, making them interesting candidates for further studies.

We have also demonstrated a novel A-P pattern in the embryonic meso-diencephalic dopaminergic domain. The diencephalic dopaminergic precursors were not affected by the loss of isthmic FGFs, whereas midbrain dopaminergic neurons required FGF signaling for their full maturation. Downregulation of FGF signaling caused midbrain dopaminergic progenitors to adopt a phenotype which molecularly resembled that of the diencephalic progenitors.

Dopaminergic progenitors in *Fgfr* compound mutants lost TH by birth. Whether this reflects a normal fate of the diencephalic dopaminergic neurons, or whether it is caused by the loss of FGFs, is at this moment unclear and would require a fate-mapping approach. Nevertheless, our observations might help to provide one piece to the big puzzle of dopaminergic neuron development. Understanding the molecular basis behind heterogeneity of different dopaminergic populations is essential for the development of therapeutic approaches to neurodegenerative diseases.

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