FIBROBLAST GROWTH FACTOR SIGNALLING IN THE DEVELOPMENT OF THE MIDBRAIN AND ANTERIOR HINDBRAIN

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ACADEMIC DISSERTATION

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Cover image: *Fgf8* whole-mount in situ hybridization in the *Cdh22^{null}* mutant embryo at E10.5

All truths are easy to understand
once they are discovered;
the point is to discover them.
Galileo Galilei

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LIST OF ORIGINAL PUBLICATIONS

I. Blak AA, Naserke T, **Saarimäki-Vire J**, Peltopuro P, Giraldo-Velasquez M, Vogt Weisenhorn DM, Prakash N, Sendtner M, Partanen J, Wurst W. Fgfr2 and Fgfr3 are not required for patterning and maintenance of the midbrain and anterior hindbrain. *Developmental Biology.* 2007 Mar 1; 303 (1):231-43.

II. **Saarimäki-Vire J**, Peltopuro P, Lahti L, Naserke T, Blak AA, Vogt Weisenhorn DM, Yu K, Ornitz DM, Wurst W, Partanen J. Fibroblast growth factor receptors cooperate to regulate neural progenitor properties in the developing midbrain and hindbrain. *Journal of Neuroscience.* 2007 Aug 8; 27 (32):8581-92.

III. Lahti L, **Saarimäki-Vire J**, Rita H, Partanen J. FGF signalling gradient maintains symmetrical proliferative divisions of midbrain neuronal progenitors. *Developmental Biology.* 2011 Jan 15; 349 (2):270-82.

IV. **Saarimäki-Vire J**, Alitalo A, Partanen J. Analysis of Cdh22 expression and function in the developing mouse brain. *Developmental Dynamics.* 2011 Aug 240 (8):1989-2001.

ABBREVIATIONS

III	oculomotor nerve
IV	trochlear nerve
5-HT	5-hydroxytryptamine, serotonin
AME	anterior mesoderm
ANR	anterior neural ridge
AVE	anterior visceral endoderm
Bmp	bone morphogenetic protein
C	cerebellum
CAM	cell adhesion molecule
Cdh	cadherin
Cdk	cyclin-dependent kinase
Cip/Kip	family of CKIs (includes p21, p27 and p57)
CKI	cyclin-dependent kinase inhibitor
CNS	central nervous system
DBH	dopamine β-hydroxylase
Di	diencephalon
DNA	deoxyribonucleic acid
DR	dorsal raphe nuclei
E	embryonic day
ECM	extracellular matrix
Erk	extracellular-signal-regulated kinase
FB	forebrain
Fgf	fibroblast growth factor
Fgfr	fibroblast growth factor receptor
FP	floor plate
G0	Gap0, phase of cell cycle, state of quiescence
G1	Gap1, phase of cell cycle
G2	Gap2, phase of cell cycle
GA	GABAergic neuron
GABA	gamma-aminobutyric acid
Gl	glutamatergic neuron
GTPaase	enzymes that hydrolyse guanosine triphosphate
HAV	histidine-alanine-valine tripeptide
HB	hindbrain
HD	homeodomain
HOX	homeobox
HSPG	heparan sulphate proteoglycan
IC	inferior colliculi
Ig	immunoglobulin-like domain
IgIII	immunoglobulin-like domain of Fgfr controlling binding specificity
IgCAM	immunoglobulin-like cell adhesion molecule
Ink4	family of CKIs (includes p15, p16, p18 and p19)
INM	interkinetic nuclear migration
IsO	isthmic organizer
ISVZ	inner subventricular zone
mDA/DA	midbrain dopaminergic neurons
M	mitosis
m	midbrain compartment
MAPK	mitogen-activated-protein kinase
MB	midbrain
Mes	mesencephalon
Met	metencephalon

MFB	medial forebrain bundle
MHB	midbrain-hindbrain boundary
Mo	motoneurons
MRF	midbrain reticular formation
Mye	myelencephalon
MZ	mantle zone
NTM	neurotransmitter
OSVZ	outer subventricular zone
OMN	oculomotor nucleus
p	prosomere
PAG	periaqueductal gray
PC	posterior commissure
PI3K	phosphoinositol 3 kinase
PLCγ	phospholipase Cγ
PP	posterior prethalamus
QAR	glutamine-alanine-arginine tripeptide
R	main restriction point in cell cycle
r	rhombomere
RA	retinoid acid
Rb	retinoblastoma
RP	roof plate
RN	red nucleus
RNA	ribonucleic acid
RRF	retrorubral field
S	synthesis (DNA), phase of cell cycle
SC	superior colliculi
SN	substantia nigra
SNpc	substantia nigra pars compacta
SNpr	substantia nigra pars reticulata
SNP	short neural progenitors
SpC	spinal cord
Tel	telencephalon
TH	tyrosine hydroxylase
VTA	ventral tegmental area
VZ	ventricular zone
ZLI	zona limitans interthalamica

In the text, mouse gene names are written in *Italics* and first letter capital, human genes *ITALICS* and all capital, protein names in Roman and first letter capital.

ABSTRACT

The embryonic midbrain and hindbrain give rise to brain stem structures and the cerebellum. The ventral midbrain and anterior hindbrain include highly important brain nuclei such as the dopaminergic substantia nigra and the ventral tegmental area, as well as serotonergic dorsal raphe neurons. These specific brain structures are affected in several disorders such as Parkinson's disease, depression, schizophrenia and drug addiction.

Between the developing midbrain and hindbrain is a signalling centre called the Isthmic Organizer. This Isthmic Organizer secretes signalling molecules, such as Wnts and Fibroblast growth factors (Fgfs). Fgf8 is able to induce midbrain and anterior hindbrain characteristics in ectopic locations, and thus Fgf8 can act as an organizer molecule. Fgf signals are mediated by Fgf receptors (Fgfr). Of the four *Fgfrs*, *Fgfr1-Fgfr3* are expressed in the nervous system. *Fgfr1* is required to maintain coherence of a slowly dividing midbrain-hindbrain boundary cell population. However, the role of Fgfr2 and Fgfr3 in the development of midbrain and anterior hindbrain is poorly understood as well as cell adhesion molecules related to the maintenance of the coherent isthmic constriction.

In this study, we elucidated the role of *Fgfr2* and *Fgfr3* during the development of the midbrain and hindbrain. We showed that loss of either *Fgfr2* or *Fgfr3* alone – or even both together – did not result in any structural abnormalities. Thus, *Fgfr1* is the major Fgf receptor in the midbrain and anterior hindbrain region. However, when *Fgfr1* and *Fgfr2,* or all three *Fgfr1, Fgfr2* and *Fgfr3* were simultaneously inactivated, the defects in the midbrain-hindbrain development were much more severe than in the *Fgfr1* mutants alone. Dorsal midbrain structures and the cerebellum were lost. Although some dopaminergic precursors appeared in the ventral midbrain, all dopaminergic neurons and several other ventral neuronal populations were lost by birth.
We showed that Fgfr cooperatively regulate cell survival, antero-posterior patterning, and the maintenance of neural progenitor properties. Loss of Fgf signalling in the ventral midbrain resulted in a thinner ventricular zone and premature neurogenesis. This was not caused by shortened cell cycle length or abnormalities in cellular polarity, cellular architecture or the orientation of mitotic spindles. Instead, loss of Fgf signalling lead to a downregulation of neural stem cell transcription factors, which allowed upregulation of proneural genes. Thus, these gene expression changes drove neural progenitors to exit the cell cycle. In addition, we showed that Fgf8 is localized in the basal membrane. Thus, Fgf signalling may maintain proliferative identity of the midbrain neural progenitors, and the cells likely receive these guiding Fgf signals through their basal processes.
Finally, we showed that an Fgf-regulated adhesion molecule *Cadherin22* (*Cdh22*) is not essential for the maintenance of the coherent compartment boundary between the midbrain and the hindbrain. Possibly, Cdh22 acts redundantly with other type II cadherins. In addition, specific expression patterns in distinct brain nuclei suggest roles for Cdh22 in the segregation of neuronal populations cooperatively with other cadherins.

In summary, these results demonstrate that Fgf signalling, and especially cooperation of the Fgf receptors, is required for proliferation, cell survival, and patterning of the neural progenitors in the midbrain and anterior hindbrain. A good understanding of developmental processes such as detailed mechanisms of signalling pathways and their regulation elucidates possibilities for therapeutic use.

1. INTRODUCTION

During brain development a relatively simple neural tube turns into both a complex structure and functionally elaborated neuronal network. To achieve this well-organized complexity, certain cell populations or brain regions are required to guide the development of neighbouring regions. These instructive regions are called signalling centres or organizers (Echevarria et al., 2003, Vieira et al., 2010). The organizers secrete signalling molecules that regulate gene expression and, thus, the growth and organization of surrounding areas. One of these signalling centres is the Isthmic organizer (IsO) which is located in the midbrain-hindbrain boundary. The IsO, and signalling molecules secreted from it, guide the growth and patterning of the whole midbrain and anterior hindbrain. Some important brain nuclei derived from this region include dopaminergic neurons, serotonergic neurons, locus coeruleus, and motoneurons of the III and IV cranial ganglia (Goridis and Rohrer, 2002, Puelles, 2007, Kiecker and Lumsden, 2012). Several neurological and psychiatric disorders, such as Parkinson's disease, depression, schizophrenia, and addiction, are associated with altered function of these neuronal populations (reviewed in Prakash et al., 2006) and, thus, studying this region is of great interest. The molecular mechanisms of initiation and progression of these disorders are not fully understood. Knowledge of how these neuronal populations originally develop and how intercellular signals regulate their induction and maintenance is necessary for understanding their diversity, function and pathology. Moreover, signalling molecules regulate, directly or indirectly, the proliferation and differentiation of developing neurons (Vieira et al., 2010). The balance between these actions ensures the maintenance of an appropriate progenitor cell pool and sufficient production of neurons. Modifications of this balance may have contributed to the expansion of brain size during evolution (Kouprina et al., 2004, Buchman and Tsai, 2007, Fietz and Huttner, 2011).

2. REVIEW OF THE LITERATURE

2.1. Early brain development

2.1.1. Basic structure of developing brain

The central nervous system (CNS) is composed of the brain and spinal cord. In the beginning of nervous system development, the embryonic brain consists of three primary vesicles: the forebrain, the midbrain and the hindbrain. As development of the central nervous system proceeds, the primary vesicles are partitioned into smaller compartments called secondary vesicles (Fig. 1 A). The forebrain develops as telencephalon and diencephalon, the midbrain as mesencephalon, and the hindbrain as metencephalon and myelencephalon. These secondary vesicles then give rise to more complex structures (Fig.1 B - D).

2.1.1.1. *Structures derived from the midbrain*

The basal division of the embryonic midbrain develops into tegmentum and the alar division into tectum (Puelles, 2007). The tectum can be further divided into two main structures: The
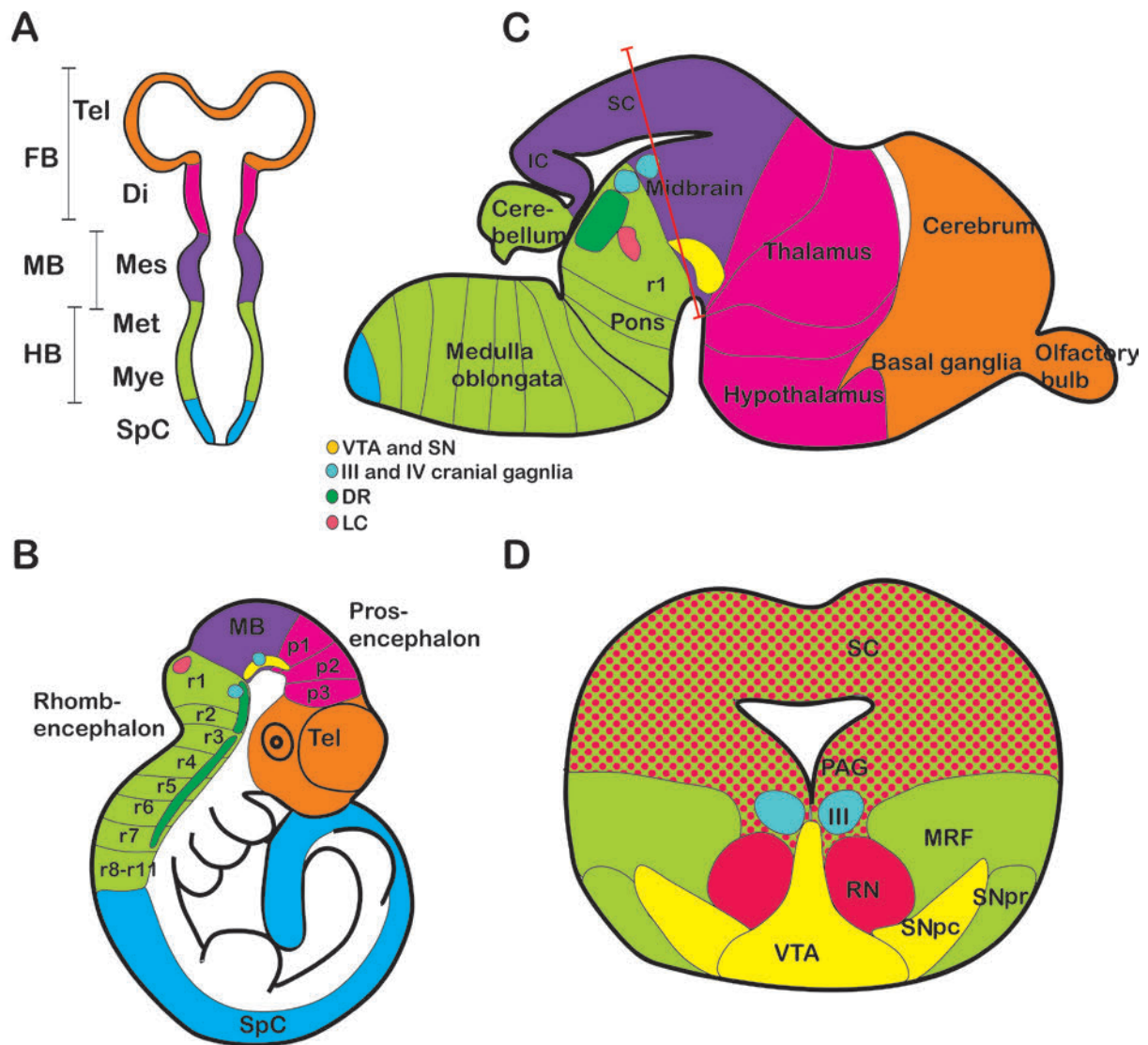
Figure 1. **Schematic view of the developing brain.** At the beginning, embryonic brain consists of three primary vesicles: forebrain, midbrain and hindbrain. This further develops into five secondary brain vesicles **(A)**: The forebrain forms the telencephalon and diencephalon. The midbrain develops as one mesencephalic compartment. The hindbrain develops into the anterior metencephalon and posterior myelencephalon. The embryonic forebrain can further subdivided as prosomeres and is, thus, called prosencephalon **(B)**. Similarly, the hindbrain is subdivided as seven rhombomeres (r1-7) and three pseudorhombomeres (r8-11) and is called rhombencephalon. At birth, these embryonic brain structures form the functional brain compartments **(C)**: The telencephalon will form the cerebrum, hippocampus, basal ganglia, and olfactory lobes. In the dorsal diencephalon, p1 develops into pretectum, p2 into thalamus and p3 to prethalamus. The ventral diencephalon develops into the hypothalamus. The ventral midbrain develops into tegmentum and the dorsal midbrain into tectum. The ventral metencephalon (r1-r3) develops into pons and the dorsal metencephalon into cerebellum. The myelencephalon (r3-r7) will form the medulla oblongata. Cross section through the midbrain at E18.5 shows organisation of certain brain nuclei **(D)**.The midbrain and anterior hindbrain give rise to brain nuclei such as dopaminergic SN and VTA, serotonergic DR, cholinergic III and IV cranial ganglia, noradrenergic LC, glutamatergic RN and GABAergic SN, MRF and VPAG associated neurons **(B - D)**. Red line in **C** marks the level of section in **D**. The brain in **C** and the borders of brain regions were drawn based on Allen Brain Atlas. FB forebrain, MB midbrain, HB hindbrain, Tel Telencephalon, Di diencephalon, Mes Mesencephalon, Met metencephalon, Mye Myelencephalon, SpC Spinal Cord, p prosomere, r rhombomere, SC superior colliculus, IC inferior colliculus, VTA ventral tegmental area, SN substantia nigra, III oculomotor nucleus, IV trochlear motornucleus, DR dorsal raphe nucleus, LC locus coeruleus nucleus, PAG periaqueductal gray, MRF midbrain reticular formation, RN red nucleus, SNpc substantia nigra pars compacta, SNpr substantia nigra pars reticulata.

anterior superior colliculi (SC) and posterior inferior colliculi (IC, Fig. 1C). The SC is associated with the function of the visual system and the IC with the auditory system. Several well-defined brain nuclei arise in the basal mesencephalic neuroectoderm (Puelles, 2007), from which some subset are described here in more detailed. The ventral midbrain gives rise to three dopaminergic neuronal populations (Fig. 1C, D): ventral tegmental area (VTA, A10), substantia nigra (SN, A9) and retrorubral field (RRF, A8). The VTA and RRF innervate the ventral striatum, which consist of accumbens nucleus, amygdala and olfactory tubercle, and limbic cerebral cortex, through mesolimbic and mesocortical pathways, respectively (Alavian et al., 2008). The function of these neuronal populations is associated with cognitive processes such as memory, association, attention and language (Prakash and Wurst, 2006a). The substantia nigra (SN, Fig. 1D) is located in the latero-ventral midbrain and diencephalon, and consists from GABAergic pars reticulata (SNpr) and dopaminergic pars compacta (SNpc) portions. SNpr GABAergic interneurons regulate the function of dopaminergic neurons, and they innervate the thalamus and the SC. The SNpc innervates dorsolateral striatum and caudate putamen forming nigrostriatal pathway, which contributes to movement control (Alavian et al., 2008). Dorsal to the VTA and the SN is located red nucleus (RN, Fig. 1D, Puelles, 2007). The RN participates in the rubrospinal tract, which controls large muscles and fine motor movements. Dorsal to the RN is located oculomotor nucleus (OMN, III cranial nerve). The OMN neurons innervate eye muscles. Periaqueductal gray (PAG) is located close the midbrain ventricle (Puelles, 2007). The PAG neurons are associated with modulation of pain and defensive behaviour. In addition, the mesencephalon includes neurons of the midbrain reticular formation (MRF, Fig. 1D), and is associated motor control patterns. In conclusion, the midbrain gives rise to a complex array of brain structures which contribute to controlling movement and behaviour.

2.1.1.2. Structures derived from the anterior hindbrain

The hindbrain is subdivided into seven rhombomeres (r). The basal metencephalon (r1-r3) develops into pons and the alar division into cerebellum (Fig. 1C). The cerebellum contains two foliated hemispheres and medial part, called a vermis. The cerebellum is a layered structure, which consists of several different cell types. Purkinje cells and deep cerebellar nuclei arise from the metencephalic alar plate. The Purkinje cells migrate along radial glial cells into cerebellar cortex (ten Donkelaar et al., 2003). Granule cell precursors originate from the upper rhombic lip, which is derived from the dorsal r1 (Wingate, 2001). Before exiting the cell cycle, the granule cell precursors migrate and accumulate in more caudal locations to form external granular cell layer. The final maturation of granule cells occurs when the external cranial cells undergo final mitosis and migrate radially to form an internal granular cell layer. The vermis of cerebellum is originated from the roof plate of the anterior rhombomere1 (Zervas et al., 2004, Sgaier et al., 2005). The cerebellum, besides other functions, controls muscular movements and balance. The trochlear motor nucleus (IV cranial ganglia) develops from the basal r1 (Goridis and Rohrer, 2002). The trochlear nerve innervates the eye muscles. The serotonergic neurons in raphe nuclei also arise from the basal hindbrain and the axons innervate all parts of the CNS. The noradrenergic Locus Coeruleus (LC) arises from the r1 alar plate, although it is finally located in the dorsal partition of the anterior pons (Goridis and Rohrer, 2002, Kiecker and Lumsden, 2012). The axons of the LC reach the other brain regions widely. The myelencephalon (r4-r7) forms the medulla oblongata (Fig. 1C), which contains neuronal centres involved in function of digestive system, heart and blood vessel activity, breathing, and reflexes.

2.1.2. Neural induction and neurulation

Neural induction. During gastrulation, the pluripotent epiblast produces three germ layers, ectoderm, mesoderm and endoderm, from which all tissues of upper animals are derived (Gilbert, 2003, Stern, 2005). During gastrulation, epiblast cells migrate through a primitive streak into the space between the epiblast and hypoblast and form first the endoderm and slightly later a medial layer, the mesoderm. The remaining epiblast forms the ectoderm. The ectoderm gives rise to an epidermis, neural crest and neural tissue (Gilbert,2003). The first experiments to introduce the concept of organizer tissues regulating embryonic development were carried out by Spemann and Mangold in 1924 (Stern et al., 2006). They transplanted a lip of the dorsal blastopore of an early amphibian gastrula into the ventral ectoderm of another gastrula. The transplanted blastopore lip was able to initiate gastrulation and the duplication of dorsal structures also in the ventral side of the embryo. The transplanted tissue appeared to induce surrounding tissue to form new secondary axis with normal tissue organization. The dorsal lip, which was able to promote a new secondary axis and correct antero-posterior and dorso-ventral organization, was named the Spemann's organizer. Later, functionally homologous organizers have been found from other vertebrate species: the shield in fish, the Hensen's node in chick, and the node in mouse (Brewster and Dahmane, 1999). These first inductive tissues are called primary organizers.

Neural induction is step-wise process, which begins before gastrulation (Stern et al., 2006). First, the ectoderm is activated by pre-neural/ pre-forebrain genes. Activating signals are secreted simultaneously by underlining mesodermal tissue, the hypoblast (chicken) or an anterior visceral endoderm (AVE, mouse) and a node. Fgf8 secreted from chicken hypoblast and mouse AVE has been suggested to be this signal, which activates pre-neural fate in the ectodermal tissue (Stern et al., 2006, Mason, 2007). Fgf signalling, either directly or indirectly by inhibiting Bmps, can regulate induction of neuronal fate. This activation causes an unstable pre-neural or pre-forebrain state to the ectodermal tissue, which then express *Erni* and *Sox3* genes. Second, neural fate is stabilized by factors, including BMP inhibitors Noggin and Chordin, segregated from the node or an anterior mesoderm (AME). Neural fate is induced, when ectodermal tissue expresses *Sox2* and *Otx2* (Stern, 2005, Levine and Brivanlou, 2007). The factors able to induce *Sox2* expression are not known. However, Wnt and Fgf signals are able synergistically to activate the *Sox2* enhancer region (Takemoto et al., 2006). Third, neural fate is caudalised by factors, such as Fgf, Wnt and Bmp inhibitors, secreted by non-axial mesoderm (Stern et al., 2006, Levine and Brivanlou, 2007). During this caudalisation period more posterior CNS structures such as the midbrain, the hindbrain and the spinal cord are formed.

According to the classical 'neural default' model, neural fate is induced by bone morphogenetic protein (Bmp) antagonists, such as Noggin, Chordin, and Follistatin, which inhibit the formation of the epidermis. However, neither Chordin nor Noggin are able to induce neural fate if Fgf signalling is blocked by dominant-negative Fgf receptors. Fgf signalling is largely accepted as a key player in neural induction at least *Xenopus*, chick, zebrafish, and ascidians (Mason, 2007). In mammals, the role of Fgf signalling in neural induction is poorly understood.

Neurulation. During neurulation, the neural plate develops into a neural tube. In higher vertebrates neurulation proceeds in two steps. First, the brain and most of the spinal cord are formed by primary neurulation. Secondary neurulation occurs caudally from the mid-sacral region of the spinal cord, when tail-bud derived mesenchymal cells condensate to form an epithelial rod in the tail bud. Inside the rod develops a canal, which fuses with the neural tube formed by primary neurulation (Greene and Copp, 2009). In primary neurulation, the neural plate is shaped as a tube. The neural plate thickens apico-basally, narrows laterally and elongates antero-posteriorly by convergent extension movements. During bending neural folds elevate to form wedge-shaped neural groove and rotate around hinge points. The neural tube closes at the dorsal midline when the neural folds fuse to form a roof of cylindrical-shaped neural tube (Colas and Schoenwolf, 2001). The neural tube closure starts first from the hindbrain-cervical boundary (at E8 in mouse) and two other closure points appear later (at E9 in mouse) in a forebrain-midbrain boundary and in the rostral end of the forebrain. From the closure points, neurulation proceeds bidirectionally to generate the neural tube (Greene and Copp, 2009).

2.1.3. Neural patterning

During patterning the antero-posterior (head-tail), left-right and dorso-ventral (back-belly) axes are determined (Gilbert, 2003). Genetically regulated signals induce expression of certain transcription factors that regulate fate specification of certain cell types. Diffusible factors, which can regulate development and identity of surrounding regions are called morphogens. The morphogens commonly form concentration gradients in the adjacent regions, being greatest near the expression source. At a certain distance, the morphogen concentration achieves a threshold which induces the formation of a certain cell type (Gilbert, 2003).

2.1.3.1. Growth factors in neural patterning

During neural patterning several growth factors act as morphogens. The Fgf (see page 29), Bone morphogenetic protein (BMP), Transforming growth factor-β (Tgf-β), Wnt, Sonic hedgehog (Shh) and Retinoid Acid (RA) signalling pathways establish molecular cascades to regulate cellular specification in the developing nervous system. Bmp and Tgf-β signalling is transduced by a signalling cascade, where the ligand binding causes dimerization of receptor type I and type II. Trans-phosphorylation of heterodimers activates the binding and phosphorylation of Smad proteins, which enter the nucleus and induce or repress target gene expression. In Wnt signalling, Wnt ligand binds to Frizzled receptor, which activates Disheveled. Disheveled prevents β-catenin degradation by inhibiting formation of the β-catenin-Gsk3-APC-Axin complex. β-catenin enters to the nucleus and associates with Lef/Tcf proteins to activate transcription factors. Shh signalling is mediated by the receptor protein Patched, which controls signal transducer Smoothened. Active Smoothened allows Gli proteins to enter the nucleus and act as transcription factors. The absence of ligand results in inhibition of Smoothened, which causes cleavage of Gli proteins changing their function into transcriptional repressors. RA is processed from vitamin A (Retinoid) by dehydrogenases (Maden, 2002). Inside the signal receiving cell, RA enters the nucleus and binds to the nuclear receptor RAR, which dimerises with retinoid X receptor (RXR) to regulate gene expression.

2.1.3.2. Antero-posterior patterning directs regional specification during axis formation

Local differences in cell fates contribute to regional specification along the antero-posterior axis. First, anterior neural fate is induced during neural induction (see above). Posterior neural fates (the hindbrain and the spinal cord) are induced by posteriorizing factors. Candidates for these posteriorizing signals are Wnts, RA and Fgfs (Maden, 2006, Stern et al., 2006, Mason, 2007). The developing brain is divided according to a prosomeric model by longitudinal and transverse boundaries (Vieira et al., 2010). The boundaries segregate cells into brain compartments that include cells with similar properties. Transversally, the forebrain or prosencephalon can be divided into the secondary prosencephalon, which includes the telencephalon and the hypothalamus, and three diencephalic prosomeres (p1-p3, Fig. 1B, Puelles and Rubenstein, 2003). The midbrain is not divided, and the hindbrain or rhombencephalon is subdivided as seven rhombomeres (r1-7) and three pseudorhombomeres (r8-11, Fig. 1B, Vieira et al., 2010). Slightly after neural induction, secondary organizers are established at several boundary regions of developing brain, and their function refines cellular specification in different neuronal compartments (Fig. 2A, Vieira et al., 2010).

Secondary organizers drive the patterning of the early embryonic brain. Patterning of brain compartments is regulated by signals secreted from signalling centres. The signalling centres or secondary organizers are often located at the compartment boundaries and can induce adjacent tissue to adopt a new fate. Three such secondary organizers have been identified from the developing brain: anterior neural ridge (ANR), zona limitans intrathalamica (ZLI), and Isthmic organizer (IsO, Fig 2A, Echevarria et al., 2003, Vieira et al., 2010).The ANR is located at the rostral-most-end of the telencephalon. Deletion of the ANR results in a failure in anterior patterning and substantial cell death in the rostral telencephalon. Signals from the ANR, such as *Fgf8* and *Shh*, are essential for specification of telencephalic neural precursors (Echevarria et al., 2003, Vieira et al., 2010, Kiecker and Lumsden, 2012). The second organizer, the ZLI forms into the diencephalic region located between p2 and p3. The signalling molecule secreted by the ZLI is Shh, which is necessary for specification of diencephalic compartments and cell fates (Echevarria et al., 2003, Vieira et al., 2010, Kiecker and Lumsden, 2012). The third secondary organizer, the IsO, is located between the midbrain and the hindbrain. As other organizers, the IsO regulates the morphogenetic properties of the midbrain and anterior hindbrain by expressing signalling molecules such as *Fgf8* and *Wnt1* (see below, Echevarria et al., 2003, Vieira et al., 2010, Kiecker and Lumsden, 2012). Organizer signals, such Shh and Fgf8, regulate the expression of transcription factors, which often belong to the Homeodomain (HD) transcription factor family. These transcription factors are induced in certain brain compartments or cellular populations, which have a certain competence to respond to organizing signals. Distinct cellular domains can, thus, be separated by specific expression of certain HD transcription factors (Vieira et al., 2010). For example, *Pax6* is expressed in the diencephalon, *Otx2* anteriorly from the midbrain-hindbrain border and *Gbx2* posteriorly from the midbrain-hindbrain border (Vieira et al., 2010). *En1* and *Pax2* are expressed throughout the midbrain-rhombomomere1 territory.

Antero-posterior patterning of the hindbrain and spinal cord. Patterning of the hindbrain and spinal cord is regulated by *Hox* genes (Lumsden and Krumlauf, 1996). The *Hox* genes are arranged in 13 paralogous groups in four clusters (*HoxA-Hox;* Luniella and Trainor, 2006). The closer a 3' end *Hox* gene is situated in the cluster, the earlier and more anteriorly it is expressed. The establishment of certain rhombomeric characteristics, and compartment boundaries between distinct rhombomeres, requires expression of *Hox* genes from paralog

groups 1-4 along the antero-posterior axis of the hindbrain. RA and FGF signalling induce *Hox* gene expression. RA is expressed as a rising gradient in the hindbrain. The 3' *Hox* genes require less RA to be activated compared to 5' *Hox* genes. Furthermore, ectopic *Fgf* signalling is able to induce 5' *Hox* gene expression in the caudal hindbrain, but not expression of the 3'*Hox* genes of 3'end. Instead, *Fgf8* secreted from the Isthmus determines the anterior limit of *Hox* gene expression (Irving and Mason, 2000). Blocking *Fgf8* in chick embryos leads to the spreading of *HoxA2* anteriorly to the r1 and loss of typical r1 characteristics. Implanted pieces of the Isthmus or beads containing Fgf8 are able to inhibit *Hox*-gene expression in the caudal hindbrain, where *Hox*-genes are normally expressed. Relatively complex cross- and autoregulatory loops are required to initiate and maintain expression of the *Hox* genes (Deschamps, 2007).

2.1.3.3. Dorso-ventral patterning of spinal cord

The longitudinal boundaries are defined by dorso-ventral patterning. The dorsal midline cell population forms the a roof plate and more lateral dorsal cells form an alar plate. The ventral midline cells form a floor plate and ventral cells more laterally form a basal plate. The dorso-ventral identity is established by opposing interaction between dorsalizing and ventralising factors (Nishi et al., 2009). Dorsalizing signals include members of Bmp and Wnt families. A ventralising factor is Shh. Shh from the floor plate and Bmps from the roof plate form a concentration gradient in the neural ectoderm and are, thus, able to induce specific gene expression and cellular domains that are committed to certain cell fates according to their gene expression profiles (Nishi et al., 2009). Bmps induce expression of the class I homeobox transcription factors: *Pax7*, *Dbx1*, *Dbx2*, *Irx3* and *Pax6* (Briscoe, 2009). Expression of these transcription factors is repressed by Shh. They have a distinct sensitivity to Shh repression, *Pax7* being most sensitive and *Pax6* least sensitive to Shh. In contrast, Shh signal induces expression of class II transcription factors *Nkx6.1* and *Nkx2.2* of which *Nkx6.1* is more sensitive to Shh signals and, thus, is induced in more dorsal locations than *Nkx2.2*. Moreover, the class I and the class II transcription factors mutually antagonize the expression of each other (Ulloa and Briscoe, 2007, Briscoe, 2009, Balaskas et al., 2012).

2.1.4. Patterning of the midbrain-hindbrain region

2.1.4.1. Formation of the Isthmic organizer

Transplantation studies carried out 20 years ago revealed that the junction between midbrain and hindbrain (Isthmus) has tissue organizing activities (Martinez et al., 1991, Marin and Puelles, 1994, Martinez et al., 1995). If tissue from the midbrain-hindbrain boundary (MHB) was transplanted to more anterior or posterior locations between the caudal diencephalon and the hindbrain or was inverted, the graft was able to maintain its own identity and induce *Engrailed2* (*En2*) expression and midbrain or cerebellar fate in surrounding tissue (Alvarado-Mallart, 1993, Alvarado-Mallart, 2005). In contrast, if anterior midbrain tissue was grafted into another region, the transplant adopted a new identity according to its host environment. Similarly, if the isthmic region was grafted outside the diencephalon-hindbrain region, ectopic midbrain and cerebellar fates were not induced in the adjacent tissue (Joyner et al., 2000). Thus, the MHB tissue has the capacity to induce midbrain and cerebellum development in competent tissue.

The first sign of developing midbrain-hindbrain border is the expression of two homeobox genes, *Otx2* and *Gbx2* (Fig. 2B), which are found in anterior and posterior portions of early (E7.5) embryo (Joyner et al., 2000). *Otx2* is expressed in the forebrain and the midbrain, and the expression has its caudal limit at the midbrain-hindbrain border. *Gbx2* is expressed in caudal regions of the developing nervous system and is rostrally restricted to the anterior border of r1. Establishment of these expression domains appears originally independently from each other, but the expression of *Gbx2* and *Otx2* genes are needed to suppress each other to establish a sharp midbrain-hindbrain compartment boundary and spatial gene expression patterns around the midbrain-hindbrain border (Broccoli et al., 1999, Millet et al., 1999, Li and Joyner, 2001). Thus, opposing interactions between Otx2 and Gbx2 are required for correct localization of the IsO and expression of *Fgf8, Wnt1,* and other MHB specific genes (Fig. 2A, Li et al., 2002). The embryos which lack Otx2 from the epiblast lose the forebrain and the midbrain structures (Acampora et al., 1995, Rhinn et al., 1998). In contrast, loss of Gbx2 results in deletion of the anterior hindbrain (r1-3, Wassarman et al., 1997, Millet et al., 1999). Overexpression of *Otx2* or *Gbx2* in the midbrain-hindbrain boundary results in the establishment of the MHB specific gene expression pattern (see Fig. 2B), but the expression is initiated in ectopic locations: misexpressed *Otx2* induces ectopic MHB gene expression in the hindbrain and ectopic *Gbx2* expression in the midbrain (Broccoli et al., 1999, Millet et al., 1999, Joyner et al., 2000). How the expression territories of *Otx2* and *Gbx2* are originally established is not fully understood. However, Fgf8, Wnt1 and RA, as well as factors secreted from non-neuronal tissues, such as Fgf4 and AVE derived molecules, have been suggested to regulate the initiation and maintenance of *Otx2* and *Gbx2* expression (Wurst and Bally-Cuif, 2001, Prakash and Wurst, 2004, Hidalgo-Sanchez et al., 2005, Nakamura et al., 2005).

2.1.4.2. The midbrain-hindbrain boundary specific genes

In addition to *Otx2* and *Gbx2*, several other genes are activated in the midbrain-hindbrain boundary (MHB) during IsO specification (Fig. 2B). The expression of the signalling molecule *Wnt1* covers initially the whole midbrain and *Fgf8* covers the r1, but their expression becomes restricted to juxtaposed narrow stripes on both sides of the midbrain-hindbrain border by E9.5 (Fig. 2A and B). The homeobox genes *Engrailed-1* (*En1*) and *En2,* as well as paired domain containing transcription factors *Pax2* and *Pax5,* are expressed early throughout the midbrain-r1 region and are later restricted to the posterior midbrain and the anterior hindbrain (Joyner et al., 2000).

Fgfs. *Fgf8*, *Fgf17* and *Fgf18* are expressed in the MHB region. Fgf8 appears to be the main signalling molecule that acts as an organizer (Crossley et al., 1996). Fgf8 soaked beads, transplanted to the diencephalon, the midbrain or the hindbrain territories were able to induce ectopic midbrain or cerebellum structures and ectopic expression of the MHB specific genes, similarly to IsO transplantation (Irving and Mason, 2000). *Fgf8* expression is induced in the *Gbx2*-expressing territory at E8.0 (Heikinheimo et al., 1994, Crossley and Martin, 1995). Two isoforms of Fgf8, Fgf8a and Fgf8b, are expressed in the midbrain-hindbrain region, and they have distinct functions (Liu et al., 1999, Sato et al., 2001a, Prakash and Wurst, 2004, Sato et al., 2004). When the isoforms were ectopically expressed under the Wnt1-enhancer, *Wnt1-Fgf8a* transgenic embryos showed an overproliferation causing enlarged midbrain and caudal diencephalon, but expression of the MHB specific genes was not affected (Lee et al., 1997). *Wnt1-Fgf8b* induced hindbrain gene (*Gbx2*) expression in the midbrain and caudal forebrain (Liu et al., 1999). These results suggest that Fgf8a has a mitogenic role during midbrain-

hindbrain development, but it lacks the organizer activity. In contrast, Fgf8b has the ability to induce expression of MHB specific genes and identity of the rostral hindbrain, indicating a role as the patterning molecule. In addition to Fgf8, Fgf17 has two isoforms, Fgf17a and b, whereas Fgf18 lacks splice variants (Olsen et al., 2006). The structure of Fgf8 subfamily proteins allows binding to c isoforms of Fgfr1-3 and to Fgfr4. Because the 3D structure of Fgfr domain controlling binding specificity (IgIII) is similar between Fgfr c isoforms, the ligand binding affinity to different receptors is quite similar (Olsen et al., 2006). However, there is variation in binding affinity between splice variants of the Fgf ligands. The Fgf8b isoform contains a specific amino acid (F32) in its N-terminus, which allows higher binding affinity to Fgfr c isoforms. Also, Fgf8a is able to bind Fgfr c isoforms, but considerably weaker than the Fgf8b isoform. The Fgf17b isoform and Fgf18 have similar amino acid residues as Fgf8b, but their binding affinity to Fgfr is at an intermediate level. The higher receptor-binding affinity of Fgf8b enables stronger receptor activity and, thus, induces greater mitogenic or organizing functions (Olsen et al., 2006, Sunmonu et al., 2011b). Similarly, the intermediate binding level of Fgf17b and Fgf18 corresponds to intermediate patterning activity.

Conditional inactivation of *Fgf8* in the mouse midbrain-anterior hindbrain region at 10-somite stage does not affect early initiation of IsO activity, but later the expression of *Wnt1*, *Gbx2*, *Fgf17* and *Fgf18* are downregulated or lost (Chi et al., 2003). The organizer activity is also lost in zebrafish *acerebellar* mutant embryos, which carry a point mutation in the *Fgf8* locus (Reifers et al., 1998, Jaszai et al., 2003).This inactivation causes loss of the whole midbrain and hindbrain indicating instructive role for Fgf8 in the development of the midbrain and anterior hindbrain region. Deletion of *Fgf8b* results in a similar phenotype as conditional inactivation of *Fgf8* indicating that Fgf8b is carrying the functional activity of Fgf8 (Guo et al., 2010). In contrast, inactivation of *Fgf8a* leads to post-natal lethality and growth delay, but defects in the midbrain and anterior hindbrain are absent. Thus, Fgf8a may have just a modulatory role in the function of the IsO. However, Fgf8a appears to be needed earlier, during gastrulation, to establish normal gene expression in the primitive streak (Guo and Li, 2007). Loss of *Fgf17* causes milder defects in the proliferation of progenitor cells in the caudal midbrain and cerebellum (Xu et al., 2000, Prakash and Wurst, 2004). However, the mutant lacks patterning defects in the MHB gene expression domain indicating that Fgf17 is not performing organizer activity in the IsO. Deletion of *Fgf18* alone does not affect the development of midbrain and hindbrain (Liu et al., 2002, Ohbayashi et al., 2002). Studies with chick embryos have also shown, that Fgf18 does not have ability to induce MHB specific gene expression (Liu et al., 2003). However, both ectopically expressed *Fgf17b* and *Fgf18* have a mitogenic effect on midbrain proliferation similarly to Fgf8a.

Wnt1. *Wnt1* is another signalling molecule expressed in the IsO as a narrow stripe, but on the midbrain side (Fig. 2A and B). Loss of *Wnt1* results in a large deletion of the midbrain-hindbrain region (McMahon and Bradley, 1990, Chi et al., 2003). This structural deletion might be caused by early downregulation of *Fgf8* and *En1* (Bally-Cuif et al., 1992, Lee et al., 1997, Prakash and Wurst, 2004). Moreover, a downstream mediator of canonical-Wnt signalling, β-catenin, appears to regulate *Fgf8*. Sustained expression of *β-catenin* causes upregulation of *Fgf8*, whereas inactivation leads to *Fgf8* downregulation (Chilov et al., 2010).
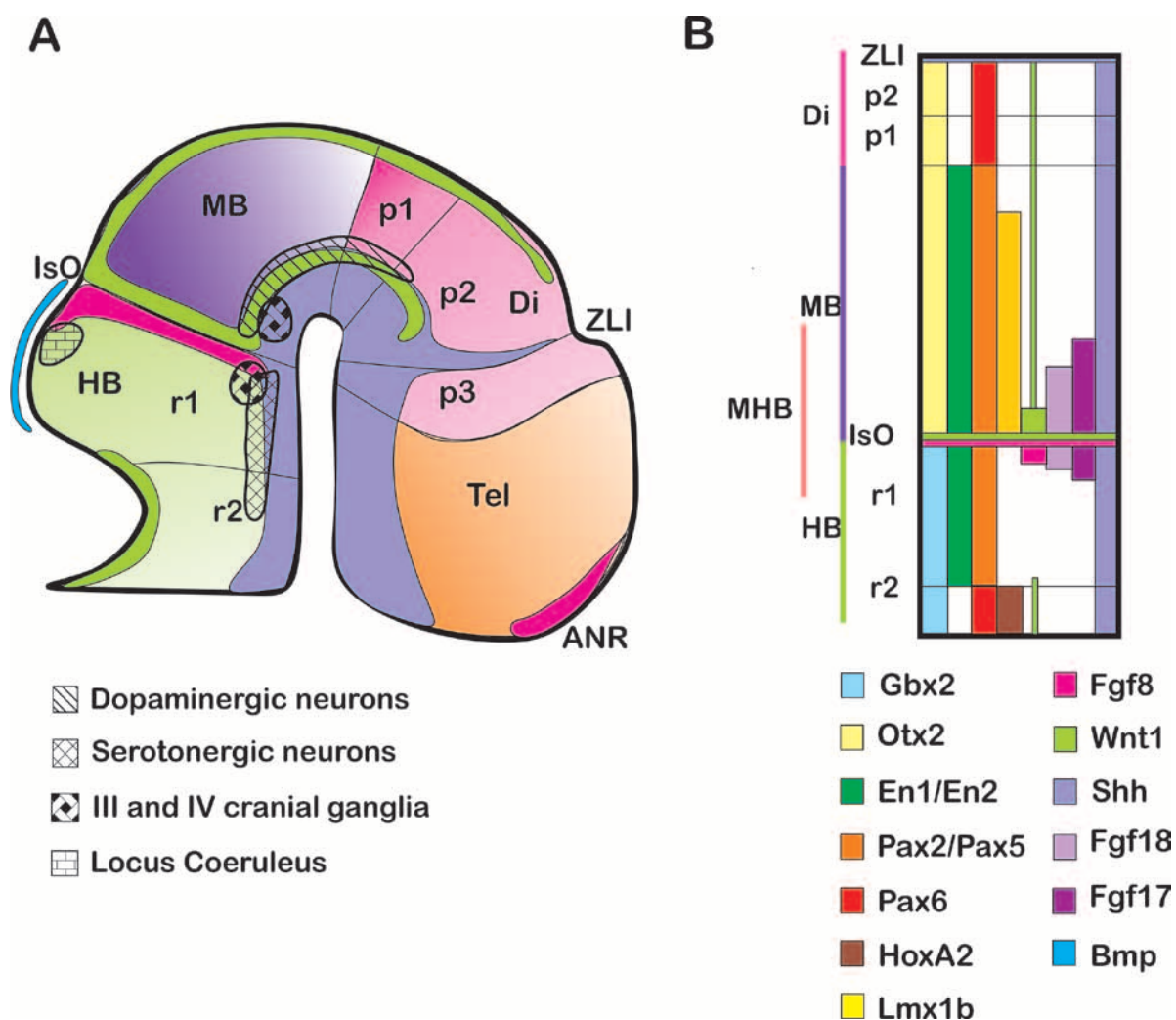
Figure 2. **Patterning of the midbrain and anterior hindbrain.** Schematic view of E10.5 embryonic brain: secondary organizers (ANR, ZLI and IsO), some neuronal populations developing in the midbrain and anterior hindbrain region, and signalling molecules affecting the development of these neuronal populations **(A)**. Dopaminergic neurons are derived from the ventral midbrain and diencephalon, motoneurons of III and IV cranial ganglia from the ventral midbrain and rhombomere1, serotonergic neurons develop in the ventral hindbrain and noradrenergic neurons of Locus coeruleus are derived from the dorsal rhombomere 1. *Fgf8* (from IsO), *Shh* (from floor plate), *Bmps* (from roof plate) and *Wnt1* (from floor plate, IsO and roof plate) instruct the patterning of these neurons. Homeodomain transcription factors and signalling molecules relating to formation of the Isthmic organizer and patterning of the midbrain and anterior hindbrain **(B)**. These genes form a midbrain-hindbrain boundary (MHB) specific gene expression pattern. Tel Telencephalon, Di diencephalon, p prosomere, MB midbrain, HB hindbrain, r rhombomere, ANR anterior neural ridge, ZLI zona limitans intrathalamica, IsO Isthmic organizer, MHB midbrain-hindbrain boundary. (Joyner et al., 2000, Wurst and Bally-Cuif, 2001, Echevarria et al., 2003, Prakash and Wurst, 2004, Sato et al., 2004, Nakamura et al., 2005, Vieira et al., 2010)

Thus, Wnt1 and Wnt signalling are needed for the maintenance of *Fgf8* and *En1* expression in the midbrain-r1. However, ectopic expression of *Wnt1* in the midbrain-hindbrain region leads to increased proliferation in the midbrain similar to Fgf8a, but is not affecting the midbrain-r1 patterning (Panhuysen et al., 2004, Prakash and Wurst, 2004). Expression of *Wnt1* might be regulated by a LIM-homeodomain gene *Lmx1b* (Fig. 2B; Adams et al., 2000, Nakamura et

al., 2005). Missexpression of *Lmx1b* in the chick midbrain-r1 causes overgrowth of the midbrain and the cerebellum, and induction of *Fgf8* and *Wnt1* (Adams et al., 2000, Matsunaga et al., 2002). In zebrafish, the absence of Lmx1b leads to deletion of the isthmic and the cerebellar structures and early loss of the MHB specific genes including *Fgf8* (O'Hara et al., 2005). Conditional deletion of *Lmx1b* in mouse embryos prevents the initiation of *Fgf8* expression and causes a failure in the maintenance of MHB specific gene expression, such as *Wnt1*, *En1*, *En2*, *Pax2* and *Gbx2* (Guo et al., 2007).

Engrailed and Pax genes. *En1* and *En2* are also expressed in the midbrain-anterior hindbrain territory from early-somite-stages (Fig. 2B). These two genes cooperate in the regulation of MHB specific gene expression, since single mutants of these genes show relatively minor defects in the midbrain-hindbrain region (Prakash and Wurst, 2004). In contrast, *En1;En2* double mutants show loss of the midbrain-hindbrain territory and early reduction of *Wnt1*, *Fgf8* and *Pax5* expression (Liu and Joyner, 2001, Prakash and Wurst, 2004). *Pax2* is the earliest gene expressed in the midbrain-hindbrain region already at presomitic stages. Deletion of *Pax2* results in an induction failure of *Fgf8* expression (Ye et al., 2001). Although expression of other MHB specific genes is initiated, the whole midbrain-hindbrain boundary is lost. *Pax5* is also expressed in the midbrain and anterior hindbrain, but loss of *Pax5* results in a relatively mild phenotype in the dorsal structures of the midbrain-r1 region. Expression of *Pax5* from the *Pax2* locus can rescue the *Pax2^null^* mutant phenotype indicating that differences in these phenotypes are a result of differences in spatiotemporal expression domains (Bouchard et al., 2000). *Pax2^-/-^;Pax5^-/-^* double mutants lack the whole midbrain and cerebellum. Therefore, *Pax* genes redundantly regulate development of the midbrain-r1 region (Prakash and Wurst, 2004). Recently it was shown that, in zebrafish, *Fgf8* needs the transcription factor *Grainy head-like 2* (*Grhl2*) for the induction of *En* expression (Dworkin et al., 2012).

2.1.4.3. Maintenance of the Isthmic organizer

After induction of the expression of signalling molecules, a positive feedback loop, in which at least *Fgf8*, *Wnt1*, *En* and *Pax* genes are involved, maintains organizer activity (Wurst and Bally-Cuif, 2001). Ectopic expression of *Pax2/5* and *En1/2* in the diencephalon induces *Fgf8*, Wnt1 and other MHB specific genes, but expression of a diencephalon specific gene, *Pax6*, is downregulated (Araki and Nakamura, 1999). Thus, Pax and En transcription factors are both downstream and upstream of IsO signals. Negative regulators, for example Sproutys in Fgf signalling and Grg4 in the case of *En* genes, suppress the organizer activity in the locations further from the isthmus. In addition, diencephalic and rhombencephalic genes, such as *Pax6* and *HoxA2*, repress MHB specific gene expression, and En1 inhibits *Pax6* and Fgf8 *HoxA2* expression (Fig. 2B; Wurst and Bally-Cuif, 2001, Hidalgo-Sanchez and Alvarado-Mallart, 2002, Nakamura and Watanabe, 2005).

2.1.4.4. Patterning and neural differentiation in the midbrain and anterior hindbrain

Fgf8, Wnt1, Shh and Bmp signals induce and regulate the expression of Homeodomain transcription factors and, thus, are key molecules in the specification of distinct cell populations in the midbrain and anterior hindbrain. Homeodomain transcription factors further induce the expression of cell lineage specific genes. The homeodomain transcription factor *Otx2* also regulates the positioning of *Shh* and *Fgf8* expression domains (Puelles et al., 2003) and is

needed for both antero-posterior and dorso-ventral specification. Deletion of *Otx2* in the midbrain causes expansion of the ventral and dorsal cell types and depression of the lateral cell types (Alexandre and Wassef, 2003, Alexandre and Wassef, 2005). Moreover, Shh signalling through activation of Gli3 maintains and, also, modulates *Fgf8* expression in the MHB (Blaess et al., 2006, Blaess et al., 2008). Fgf signalling controls the function of TgF-β family members in the dorsal midbrain and r1 allowing more complex dorsal patterning compared to the spinal cord (Alexandre et al., 2006). Thus, distinct concentrations and combinatory interactions of these signalling factors determine the future cellular identity of specific cell populations in the midbrain and r1.

Cellular specification in the midbrain. The midbrain develops from a part of neuroectoderm, which early expresses homeodomain transcription factors *Otx2*, *Pax2* and *En1* (Nakamura et al., 2005). The developing midbrain is divided into dorso-ventral domains (Fig. 3A; Nakatani et al., 2007, Kala et al., 2009). A Shh morphogen gradient induces distinct homeodomain gene expression a certain distance from the Shh source (Fig.3B; Puelles, 2007). The ventricular zone of the ventral-most domain (m7) expresses Shh, induces the expression of *Lmx1a,* and will give rise to dopaminergic (tyrosine hydroxylase (TH) expressing) neurons of VTA and SNpc. Adjacent to the *Shh*-positive domain is m6, it expresses *Nkx6.1*, and it will give rise to both *Islet1*-positive cholinergic motoneurons and *Pou4f1* positive glutamatergic neurons. The motoneurons form the oculomotor nuclei (III cranial ganglia), whereas *Pou4f1*-positive neurons form the red nucleus. However, the red nucleus cells are specified later than motoneurons. Progenitors in the next domain, m5, express both *Nkx6.1* and *Nkx2.2*. The postmitotic precursors derived from m5 induce *Gata2* expression and these cells will give rise to GABAergic neurons. The progenitors in m4 also express *Nkx2.2* and they can be subdivided into two populations: ventral and dorsal. Postmitotic precursors derived from this domain express different genes. Ventral domain expresses *Pax6*, whereas dorsal domain expresses *Gata2* (Kala et al., 2009) and they are differentially specified as glutamatergic neurons and GABAergic neurons, respectively. Progenitors from m3 express *Nkx6.1,* and after exiting cell cycle activate *Gata2* and develop into GABAergic neurons. The most dorsal domains (m2 and m1) produce heterogenic populations of both glutamatergic and GABAergic neurons (Fig. 3).

Cellular specification of the anterior hindbrain. The cerebellum arises from the *Gbx2* positive neural tube, where *HoxA2* is not expressed (Wingate, 2001). In r1, high *Fgf8* signal in the developing neural tube induces *Irx2* expression through Ras-Erk activation. The *Irx2* expression leads to the formation of the cerebellum (Matsumoto et al., 2004, Nakamura et al., 2005). Thus, the cerebellum develops from a region that receive strong *Fgf8* signal. Also in the r1, signalling molecules induce the expression of transcription factors, which specify the distinct neuronal populations. Serotonergic (5-hydroxytryptamine (5-HT+)) neurons are derived from the basal plate of the hindbrain, and can be divided into rostral (r1-3) and caudal (r3-8) divisions. The location in the basal plate ensures high concentrations of Shh (Fig. 2A) This promotes *Nkx2.2* expression in the serotonergic progenitors. The rostral serotonergic neurons receive high amount of Fgf8. Inhibition of Fgf signalling causes a loss of rostral, but not caudal, serotonergic neurons (Goridis and Rohrer, 2002). Similarly, only rostral serotonergic neurons are lost in the *Fgfr1^{cko}* mutants (Jukkola et al., 2006). *Fgf4*, which is expressed near
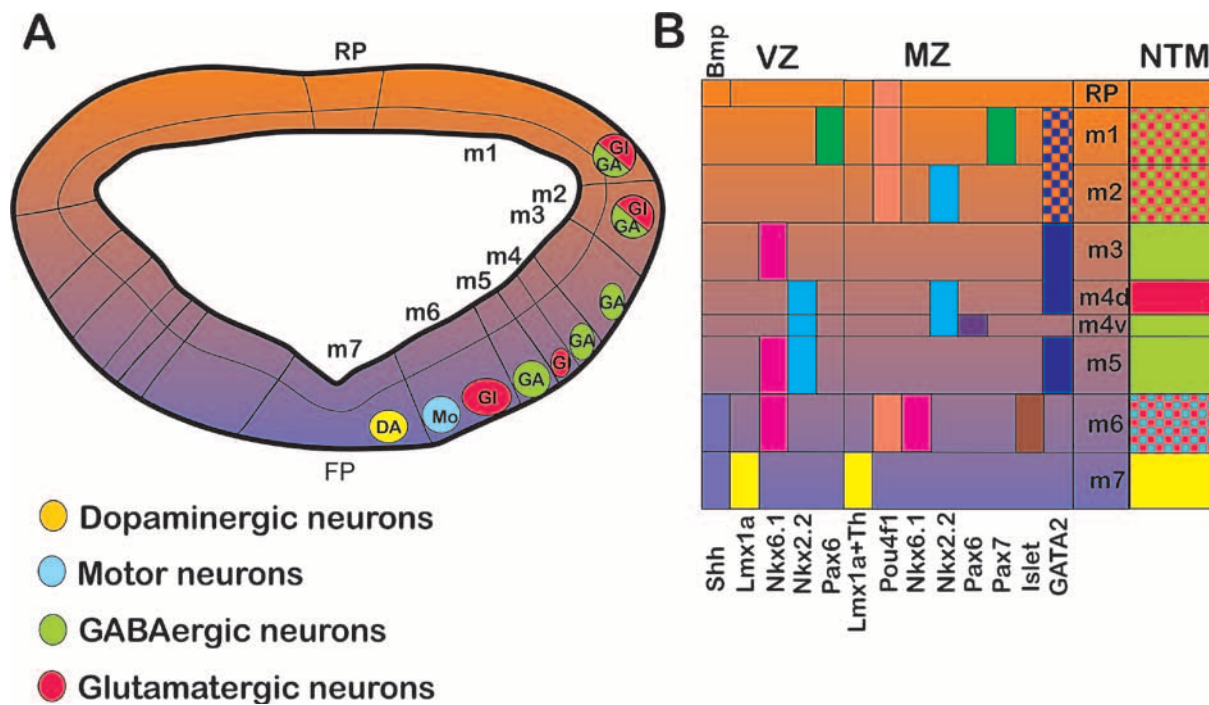
Figure 3. **Dorso-ventral domains of the developing midbrain.** Schematic view of the embryonic midbrain at E12.5 **(A)**. Embryonic midbrain can be divided as domains (m1-m7) based on expression of certain transcription factors. These transcription factors induce development of distinct neuronal populations. Shh secreted from the floor plate and Bmps secreted from the roof plate initiate the expression of certain homeodomain transcription factors in the proliferative progenitors (VZ, **B).** These transcription factors regulate expression of neuron lineage specific genes, which are typical for certain neuron population and are activated in the postmitotic neural precursors (MZ). *Lmx1a* is expressed in the dopaminergic progenitors (m7) and with *Th* also in the precursors. *Nkx6.1* is expressed in the glutamatergic progenitors (m6) and with *Pou4f1* in the precursors. The cholinergic progenitors (m6) also express *Nkx6.1*, but instead of *Pou4f1* the precursors express *Islet1*. GABAergic progenitors express different homeodomain transcription factors, but all GABAergic precursors express GATA2. RP roof plate, FP floor plate, m midbrain domain, DA dopaminergic neurons, Mo motoneurons, Gl glutamatergic neurons, GA GABAergic neurons, VZ ventricular zone, MZ mantle zone, NTM neurotransmitters. (Nakatani et al., 2007, Kala et al., 2009)

serotonergic precursors and is missing from the midbrain, has been suggested to be a factor that specifies serotonergic fate in the hindbrain (Ye et al., 1998, Goridis and Rohrer, 2002). The postmitotic precursor of serotonergic neurons expresses *Pet1*, *Gata3* and *serotonin* (Goridis and Rohrer, 2002). Trochlear motoneurons (IV cranial ganglia) are also specified in the ventral r1. These progenitors express *Nkx6.1* and precursors *Islet1* (Prakash et al., 2009). Progenitors of the LC neurons are born in the dorsal r1, and they express *Phox2a* and *Phox2b* (Goridis and Rohrer, 2002). They are specified in an environment where they receive high concentrations of Fgf8 from the IsO and Bmps from the roof plate (Fig. 2A). Postmitotic LC precursors mature and start to express TH and Dopamine β-hydroxylase (DBH, Goridis and Rohrer, 2002).

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2.1.5. Early development of the dopaminergic neurons

In the mature brain, dopaminergic neurons of the SN-VTA complex are located both in the midbrain and diencephalon. Thus, these neurons which use dopamine as a neurotransmitter have been called meso-diencephalic dopaminergic neurons (Fig. 1B and C; Smits et al., 2006). Also, there are some Th expressing precursors in the embryonic diencephalon in addition to the midbrain (Marin et al., 2005). However, recent studies have demonstrated differences in midbrain – and diencephalon – derived TH expressing precursors (Lahti et al., 2012). Thus, the following sections will focus elucidating the developmental processes involving in the development of the midbrain dopaminergic neurons (mDA).

Induction of dopaminergic fate. The midbrain dopaminergic neurons are derived from the floor plate, the ventral-most cells of the midbrain (m7, Fig. 3A). Development of the mDA neurons are induced by Shh secreted from the floor plate and Fgf8 released from the IsO (Fig. 2A; Prakash and Wurst, 2006b, Jaeger et al., 2011). Ectopic expression of *Shh* and *Fgf8* is able to induce ectopic mDA specification (Ye et al., 1998). *Shh*^{null} mutants lack the mDA progenitors, and the mDA neurons are lost from *Fgf8*^{cko} mutants at E17.5 (Chi et al., 2003, Blaess et al., 2006). *Wnt1* also appears to promote the mDA identity (Prakash and Wurst, 2007). *Wnt1* is expressed in the roof plate of the dorsal midbrain, in the IsO and in the floor plate of the ventral midbrain. Ectopic expression of *Wnt1* in the r1 induces ectopic mDA fate in the ventral r1 (Prakash et al., 2006). *Wnt1* is able to induce ectopic *Otx2* expression, which further inhibits *Nkx2.2* expression allowing induction of the DA fate. Furthermore, ectopic *Shh* and *Fgf8* are unable to induce the mDA fate if *Wnt1* expression is lacking (Prakash and Wurst, 2007).

Specification of the dopaminergic progenitors. The first molecules expressed in the dopaminergic progenitors are *Lmx1a, Lmx1b,* and *Msx1* (Andersson et al., 2006b, Alavian et al., 2008). Ectopic expression of *Shh* appears to induce expression of these genes (Andersson et al., 2006a), and induction of *Lmx1a* appears through *FoxA2* activation (Ferri et al., 2007). In chick, RNA interference of *Lmx1a* caused marked reduction of the mDA progenitors. *Lmx1b* is expressed in a broad domain already before E9. It is expressed together with *Lmx1a* and *Msx1* but alone it is unable to induce the DA fate. At early stages, *Lmx1b* plays a role in the specification of the MHB and establishment of the IsO. Later, *Lmx1a* and *Lmx1b* cooperatively regulate mDA progenitor proliferation, specification, and differentiation (Yan et al., 2011). Moreover, *Lmx1a* is able to activate the expression of *Msx1* (Smidt and Burbach, 2007). *Msx1* alone is not sufficient to induce mDA fate (Andersson et al., 2006b). However, in *Msx1*^{null} mutants the number of mDA neurons is reduced by 40%. *Msx1* represses the expression of *Nkx6.1* in the domain adjacent to the ventral midbrain (m7, Fig. 3A) and, thus, is needed to define the limits for mDA progenitor domain (Prakash et al., 2009). Moreover, *Lmx1a* and *Msx1* expression induce the expression of a proneural gene, *Ngn2*.

Differentiation of the dopaminergic precursors. After induction mDA identity, the proliferative progenitors exit the cell cycle and become postmitotic. The cell-cycle exit and neurogenic differentiation of the mDA progenitors is regulated by *Ngn2* (Andersson et al., 2006a). *FoxA1* and *FoxA2* redundantly regulate *Ngn2* expression (Ferri et al., 2007). Loss of *Ngn2* results in a reduction of mDA precursors to 20% (Kele et al., 2006). However, partial recovery of mDA numbers, likely mediated by Ascl1, occurs in later developmental stages and this recovery

fails if expression of *Ascl1* is also abolished. Overexpression of *Ngn2* increases neurogenesis, but is not sufficient to induce the mDA fate.

The mDA precursors migrate to their final positions after exiting the cell cycle. First, cells migrate radially towards the pial surface and then move laterally to populate their final destinations. Lateral neurons form the SNpc and they are thought to originate from more antero-lateral progenitors, whereas the postero-medial mDA progenitors form the VTA in the middle of the midbrain (Prakash and Wurst, 2006a, Joksimovic et al., 2009).

Several transcription factors involved in terminal differentiation and maintenance of dopaminergic fate are expressed after the cell cycle exit (Alavian et al., 2008). Alcohol dehydrogenase Aldh1, an enzyme involved in RA synthesis, is expressed in the mDA progenitors indicating a role in the early specification of the mDA fate (Smidt and Burbach, 2007). Nurr1 has been suggested to function downstream of RA signalling. Expression of *Nurr1* begins simultaneously with neurogenesis (Alavian et al., 2008). Deletion of *Nurr1* results in a maturation failure of the dopaminergic neurons (Alavian et al., 2008). In the *Nurr1* mutants, the ventral midbrain cells fail to express tyrosine hydroxylase. However, the immature mDA precursors in the *Nurr1* mutants express the earlier markers such as *Pitx3*, *En1*, *En2*, *Lmx1b* and *Aldh1*. *Pitx3* is expressed in the ventral midbrain slightly after *Nurr1* (Alavian et al., 2008). Inactivation of *Pitx3* causes the loss of the SN neurons and a portion of the VTA dopaminergic neurons (Smidt et al., 2004). Together with *Nurr1*, *Pitx3* might directly regulate expression of genes needed for dopamine synthesis and, indeed, the *Th* promoter includes a high-affinity binding site for Pitx3 (Maxwell et al., 2005, Alavian et al., 2008). Furthermore, Pitx3 appears to be an essential modulator of *Nurr1* In the regulation of specific genes for the mDA neurons (Jacobs et al., 2009). *En1* and *En2* are expressed in the mDA precursors, and their expression is needed for cell survival and the maintenance of the mDA fate (Smidt and Burbach, 2007). In *En1^{null}*;*En2^{null}* mutant mice, immature DA neurons appear at E11 and start tyrosine hydroxylase expression (Alavian et al., 2008). However, soon after this the cells die apoptotically being totally lost by E14 (Alberi et al., 2004). *FoxA1* and *FoxA2* regulate *Nurr1* and *En1* expression and, thus, are needed for both early mDA differentiation as well as maturation of mDA (Ferri et al., 2007). Abolishment of *Lmx1b* expression results the loss of TH-expressing mDA precursors by E12.5 (Alavian et al., 2008). Also, *Wnt1* is needed for maturation of the mDA precursors. The *Wnt1^{null}* mutants generate immature precursors, but the cells fail to mature for transmitter-expressing mDA neurons. Loss of *TGF-βs*, which normally are expressed from the midbrain floor plate, reduces the number of the mDA neurons by 30 % (Roussa et al., 2006). None of these factors alone is sufficient to induce the mature mDA fate and, thus, cooperation between different regulatory networks is needed to accomplish terminal differentiation of the mDA neurons.

In summary, terminal differentiation and maintenance of the mDA neurons enables the initiation of dopamine synthesis and release of neurotransmitter. As a mark of dopamine synthesis and metabolism, several proteins, such as TH, vesicular monoamine transporter 2 (Vmat2), dopamine transporter (DAT) and receptor tyrosine kinase (Ret) appear to be expressed in mature mDA neurons (Smidt and Burbach, 2007).

Connections of the dopaminergic neurons. Outgrowing axons of the mDA neurons appear to navigate through the medial forebrain bundle (MFB), when they fasciculate towards targeting regions in the basal forebrain (Smidt and Burbach, 2007). This MFB is divided into three main pathways: Axons from the SN innervate to the dorsolateral striatum, caudate and putamen, through the nigrostriatal pathway (Smidt and Burbach, 2007, Van den Heuvel and Pas-

terkamp, 2008). These axons follow the most dorsolateral path of the MFB (Hammond et al., 2009). Axons from the VTA innervate the ventromedial striatum, nucleus accumbens, and to extrastriatal structures, such as the pallium and subthalamus, through ventromedial meso-limbic and mesocortical pathways, respectively (Smidt and Burbach, 2007, Van den Heuvel and Pasterkamp, 2008, Hammond et al., 2009). The dopaminergic axons grow first out from their nuclei and turn anteriorly to enter into the diencephalon (Dugan et al., 2011). In the diencephalon, axons of the mDA neurons navigate through specific dorso-ventral positions towards their forebrain target regions. The MFB bypass the thalamus ventrally from diencephalic DA population A13 and avoid the ventrally locating hypothalamus (Dugan et al., 2011). Shh acts as a chemoattractant for these projections (Hammond et al., 2009). Specifically the most medial mDA axons are lost when expression of *Shh* in the ventral midline is abolished. Therefore, the medial and lateral axons have a distinct specificity for chemoattraction. Thus, *Shh* also provides positional information for fasciculating axons. Furthermore, Fgf8 secreted from the IsO is able to induce expression of semaphorin 3F, which as a chemorepellant controls directed growth of mDA axons towards the diencephalon (Yamauchi et al., 2009). Also, Wnt5a regulates axon elongation and pathfinding (Blakely et al., 2011). Slit-Robo signalling controls outgrowth of first pioneer axons and is involved in establishment of the MFB (Dugan et al., 2011). The ventral boundary of the MFB is regulated by Slit/Robo signalling and dorsally the MFB is restricted based on Slit/Robo and Semaphorin/Nrp2 signalling. Ventrally, Shh and Netrin signals act as chemoattractants and, likely promote axon growth. These multiple signals regulate axon outgrowth, pathfinding and connection into targeting areas (Dugan et al., 2011).

2.1.6. Neuronal progenitors and their differentiation

2.1.6.1. Cell biology of neural progenitors

Before neurogenesis, the neuroepithelium consists of a single cell layer of neuroepithelial progenitor cells (Fig. 4A). This proliferating cell layer is called the ventricular zone. When neurogenesis begins, a neural progenitor becomes a post-mitotic neural precursor and moves away from the ventricular zone. These cells form the intermediate zone. The fate of these intermediate progenitors is determined by the transcription factors they express. Mature neurons form a mantle zone near the pial surface. During neurogenesis, the neuroepithelium forms as a multi-layered structure of which the forebrain is the most complex containing six cortical cell layers.

The apical surface. The neuroepithelial progenitor cells are polarized and they have an apical end-foot towards the ventricular surface and a basal end-foot, which is connected to the pial surface (basal lamina) through a basal process (Fig. 4A). The apical plasmamembrane can be identified by the expression of cholesterol-interacting pentaspan membrane protein, Prominin1 (CD1333). Prominin1 is expressed in microvilli and during neurogenesis in the primary cilium. The primary cilium operates as an antenna, which receives extracellular signals, such as Shh and Wnt, from the ventricular surface (Fietz and Huttner, 2011). Moreover, a basal body of the primary cilium acts as an anchoring site of the centrosome, as one of the interphase centrioles participates in the basal body formation. This causes apical localization of the centrosomes in the apical progenitors (Farkas and Huttner, 2008). The neuroepithelial cells are linked together by a belt-like fashion through adherent junctions (Fig. 4A). These

consist of cell adhesion molecules, cadherins (Cdhs), connected to the cytoskeleton by catenins (Barth et al., 1997). N-Cdh and tight junction related protein Zo1 are also expressed in the adherent junctions (Aaku-Saraste et al., 1996). These apical constituents also include par complex proteins Par3, Par6 and aPKC (PKCλ and PKCζ) (Farkas and Huttner, 2008). PKCλ and a Par complex regulator, small Rho-GTPase Cdc42 have shown to be required to maintain the coherence of the adherent junctions and the polarity of the neuroepithelium (Cappello et al., 2006, Imai et al., 2006). Furthermore, the Notch inhibitor Numb protein has been shown to accumulate in the apical cell cortex and have a role in the maintenance of the adherent junctions and cell polarity (Rasin et al., 2007).

Basal connection through the basal process. The basal lamina is also required to establish polarity in the epithelial cells and is formed from a thin layer of extracellular matrix (ECM). The ECM consists of type IV collagen, nidogen and proteoglycans (Kosodo and Huttner, 2009). The signalling between the ECM and the basal process is mediated through integrin receptors and, possibly, by growth factor signalling. The mouse mutants, which have lost the contacts between the basal lamina and basal process, lack failures in proliferation or differentiation capacity; however, neuronal migration and formation of the cortical layers are disturbed (Haubst et al., 2006). More recent studies suggest that these contacts might also have a role in cortical proliferation and layer formation (Belvindrah et al., 2007, Giros et al., 2007). The basal process maintains its connection to the basal lamina also during mitosis (Miyata et al., 2001). During cell division the basal process can be inherited symmetrically or asymmetrically by daughter cells (Kosodo et al., 2008). Recent time-lapse-microscopy studies in zebrafish have shown that inheritance of the basal process is needed for the proliferative progenitors (Konno et al., 2008, Alexandre et al., 2010).

Multiple types of the neuronal progenitors. The apical progenitors are bipolar and contain both the apical- and the basal-end feet. They are connected to other apical progenitors through the adherent junctions. First apical progenitors are neuroepithelial cells and at the beginning of neurogenesis they turn into radial glial cells. The nucleus of these apical progenitors undergoes an interkinetic nuclear migration (INM, Fig. 4A, see below) and mitosis occurs adjacent to the ventricular surface (Farkas and Huttner, 2008, Fietz and Huttner, 2011). The radial glial cells express some astroglial marker genes, such as Glast and Gfap, and the basal process reaches the basal lamina through the post-mitotic cell layers although the nucleus is cycling within the ventricular zone during the INM (Gotz and Huttner, 2005). Monopolar progenitors are connected either to the ventricular surface (short neural progenitors (SNP)) or to the pial surface (outer-subventricular-zone (OSVZ) progenitors, Fietz and Huttner, 2011). The SNPs resemble the apical progenitors and do not express the basal progenitor marker gene, Tbr2, but they have a longer cell cycle similar to the basal progenitors. (Gal et al., 2006, Stancik et al., 2010). Nonpolar progenitors, such as basal progenitors and inner-SVZ (ISVZ) progenitors, lack polarity and connections to surfaces (Fietz and Huttner, 2011). The basal progenitors delaminate from the ventricular surface and are relocated more basally for mitosis. According to current knowledge, the basal progenitors occur in the subventricular zone of the mammalian cortex. The OSVZ progenitors and the ISVZ progenitors are found mainly in primate neocortex. Development of novel types of neural progenitors may explain the evolution of the primate brain size. The telencephalon of non-mammalian vertebrates and other parts of the CNS lack the SVZ structure.

2.1.6.2. The interkinetic nuclear migration and the cell cycle

The nucleus of the neuroepithelial and the radial glial cells oscillates from the apical to the pial side during the cell cycle (Fig. 4A and B). Thus, the neuroepithelial cells form a pseudo-stratified single cell layer (Farkas and Huttner, 2008). The developmental function of the INM is not fully understood, but it has been suggested to maximize the number of mitoses in limited apical surface and, thus, allow efficient progenitor expansion (Del Bene, 2011). Mitosis (M-phase of cell cycle) occurs next to the ventricular surface. During G1 the nucleus migrates from the apical surface to the pial side. In the G1-phase, the cell controls the integrity of DNA before replication (Dehay and Kennedy, 2007). Adjacent to the pial surface, DNA is replicated (S-phase) and during the G2-phase the nucleus travels back to the apical surface for mitosis (Gotz and Huttner, 2005). In the G2-phase, the cell ascertains the accomplishment of DNA replication. The major restriction point of the cell cycle appears in late G1. At this point a cell decides, whether it continues in or withdraws from the cell cycle (Dehay and Kennedy, 2007). The cells that exit from the cell cycle enter into G0-phase. The cell cycle and the INM are tightly connected in the apical progenitors. Chemical arrest of the cell cycle in the S-phase or the G2/M-phase causes a disruption of the INM movements (Ueno et al., 2006). The INM movements are suggested to be carried out by microtubule and actomyosin-based mechanisms (Taverna and Huttner, 2010). In the first, the microtubule minus end is connected the centrosome located adjacent to the apical surface. The nucleus is carried along the microtubule rails by a dynein-based trafficking mechanism in a basal-to-apical direction during the G2 phase and a kinesis-based mechanism in an apical-to-basal direction during G1. In the second mechanism, both basal-to-apical movements during G2 and apical-to-basal movements during G1 are carried by directional actomyosin constriction. Recent studies have shown that the nuclei move apically during G2 by a cell-autonomous mechanism, in which microtubule-associated protein Tpx2 alters apical microtubule organization and, thus, drives nuclear migration actively forward (Kosodo et al., 2011). In contrast, basal migration in G1 is mainly passive, a non-autonomous displacement effect caused by the adjacent G2-phase nuclei. Disruption of an F-actin polymerizing factor, N-cofilin, in the developing cortex induces increased cell cycle exit and premature neurogenesis and, thus, disruption of the microfilaments causes changes also in the cell cycle progression (Bellenchi et al., 2007). Moreover, the primary cilia, which are located at the apical surface, are connected to the cell cycle phases. In G2 the primary cilia are disassembled and re-established again after mitosis in G1 (Farkas and Huttner, 2008).

The cell cycle length hypothesis. The cell cycle length increases from 8 to 18 hours in the developing mouse cortex between E11 and E16 (Lange and Calegari, 2010). This lengthening is mainly caused by lengthening of the G1-phase from 3 to 12 hours. During G1 the cell is exposed to external cues or fate determinants which enable cell cycle re-entry or exit (Dehay and Kennedy, 2007). According to the cell cycle length hypothesis, the length of the cell cycle is suggested to determine whether a progenitor cell produces two new progenitors, a progenitor and a neuron, or two neurons after division (Calegari and Huttner, 2003). Experimental lengthening of the G1 phase of the cell cycle causes lengthening of the cell cycle and increased neurogenesis (Calegari et al., 2005, Salomoni and Calegari, 2010). *Tis21* expressing asymmetrically dividing cells, which produce one neuron and one progenitor, have a markedly longer cell cycle length than the neuroepithelial cells, which produce two progenitors (Calegari et al., 2005). A certain dosage of fate determinant at a certain time point may cause a switch from proliferation to neurogenesis. How can lengthening of the G1 phase affect
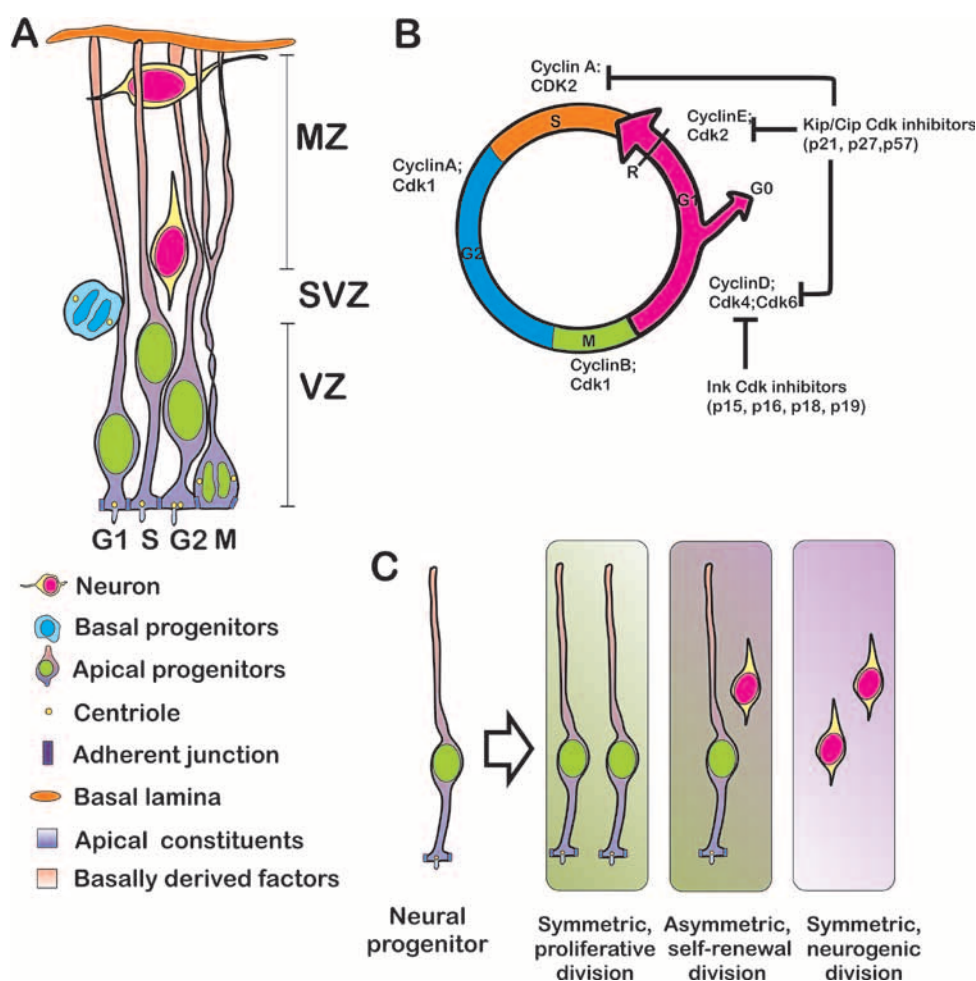
Figure 4**. The interkinetic nuclear migration and the cell cycle regulation in the neural progenitors.** The neural progenitor cell performs the interkinetic nuclear migration during the cell cycle **(A)**. The apical progenitors are attached into the apical surface and the basal lamina. The mitosis occurs adjacent to the ventricular surface and DNA replication on the basal side of the ventricular zone. The basal progenitors lack the apical contacts and divide either on the basal side of the ventricular zone or in the subventricular zone. The postmitotic neurons or the neural precursors migrate out from the ventricular zone to populate the mantle zone or cortical layers. Cyclins and Cyclin-dependent kinases (Cdks) regulate transition from one cell cycle phase to another in correct order **(B)**. CyclinD-Cdk4/Cdk6 complex controls G1 progression during early and mid G1. Cyclin E-Cdk2 complex regulates late G1 and G1-S transition. CyclinA-Cdk2 complex controls S-phase progression, and CyclinA-Cdk1 complex G2 phase. CyclinB-Cdk1 controls progression of mitosis. Cdk inhibitors (CKIs) control the function of CDKs. Ink4 CKIs (p15, p16, p18 and p19) regulate Cdk4 and Cdk6. Cip/Kip CKIs (p21, p27 and p57) regulate cell cycle regulators, such as CyclinD-, CyclinE- and CyclinA-dependent kinases, more broadly. At the G1-S restriction point, a cell decides whether to re-enter or withdraw from the cell cycle. The neural progenitor can proliferate through symmetric or asymmetric division **(C)**. Symmetric proliferative division produces two proliferative progenitors. Asymmetric, self-renewal division produces one proliferative progenitor and one neuron. Symmetric, neurogenic division produces two post-mitotic neurons. VZ ventricular zone, SVZ subventricular zone, MZ mantle zone, G1 nucleus migrate from the apical to basal side (phase of cell cycle), S DNA replication (phase of cell cycle), G2 nucleus migrate from the basal to apical side (phase of cell cycle), M mitosis, G0 quiescent state of cell (cell is not cycling in the cell cycle), R main restriction point. Cdk cyclin dependent kinase, CKI cyclin dependent kinase inhibitor. **A** is modified from (Taverna and Huttner, 2010) **B** is modified from (Dehay and Kennedy, 2007). Other references (Gotz and Huttner, 2005, Farkas and Huttner, 2008, Fietz and Huttner, 2011)

the balance between proliferation versus differentiation? Lengthening of the exposure time to a fate determinant may cause a similar result than a bigger dosage of the determinant (Calegari and Huttner, 2003, Salomoni and Calegari, 2010). The G1 phase is critical during the cell cycle and it has been shown that lengthening G1 in the developing neocortex is primarily associated with a fate switch from apical progenitors to basal progenitors (Arai et al., 2011). Moreover, a longer S phase was detected in progenitors committed to produce a new progenitor compered to progenitors that were committed to neurogenesis, suggesting that proliferative progenitors need more time for quality control than neurogenic progenitors (Arai et al., 2011).

2.1.6.3. Cell cycle regulation in neurogenesis

Cell cycle regulators. Cyclins and Cyclin-dependent kinases (Cdks) regulate the transition from one cell cycle phase to another in correct order (Fig. 4B, Dehay and Kennedy, 2007). Certain cyclins are expressed in specific phases of the cell cycle and they form complexes with certain Cdks to activate them (Fig. 4 B). The functions of Cdks are controlled by Cdk inhibitors (CKIs). Two major families of CKIs, Ink4 and Cip/Kip, regulate Cdks by separate mechanisms. At the G1-S restriction point, Cdks phosphorylate the tumour suppressor gene *retinoblastoma* (*Rb*), which causes the release of E2F from Rb-E2F complex (McClellan and Slack, 2006). This complex occurs in conditions that inhibit cell cycle re-entry. Released E2F induces genes required for DNA synthesis as well as genes for cell cycle regulation, such as *CyclinD1*, *A* and *E- myc*, *b-myb* and *Rb* family members.

Overexpression of *CyclinD1/Cdk4* shortens the G1-phase, induces proliferation and inhibits neurogenesis. Downregulation of these genes caused opposite defects including increased neurogenesis (Lange et al., 2009). Similarly, abolishing *CyclinE/Cdk2* activity lengthened G1, which elevated the rate of neurogenesis (Calegari and Huttner, 2003). Moreover, maintenance of *CyclinD1* and *CyclinD2* expression by Fgf and Shh signalling promotes proliferative divisions in chick spinal cord (Lobjois et al., 2004). CKI p27, an inhibitor of the Cdk2-CyclinE, delays the G1-S transition, inhibits cell cycle progression and promotes neurogenesis in the developing cortex (Nguyen et al., 2006). Similarly, deletion of *p21*, another inhibitor of Cdk2-CyclinE complex, causes an expansion of stem cells in the adult CNS (Kippin et al., 2005). Deletion of tumour suppressor *p53* causes increased proliferation in the developing brain (Armesilla-Diaz et al., 2009). Cumulative evidence suggests that, whenever manipulation of cyclins/Cdks causes shortening or lengthening the G1-phase, it also decreases or increases neurogenesis, respectively (Lange and Calegari, 2010). If manipulation does not affect the length of the G1, usually no effects in differentiation rate are observed. However, disruption of any single cell cycle regulator gene alone does not result in a severe defect during foetal development and, thus, none of these genes is completely essential for cell cycle progression (Sherr and Roberts, 2004).

Signalling molecules regulating cell cycle regulators. The temporal and spatial expression of cell cycle regulators is controlled directly or indirectly by signalling molecules, such as Notch, Wnt, Fgfs, and Shh (Salomoni and Calegari, 2010). Wnt signalling has mitogenic functions in the developing brain and upregulated *Wnt* expression causes elevation of *CyclinD1* levels, shortening of the cell cycle length and increased proliferation in neural progenitor cells (Panhuysen et al., 2004). Stabilized expression of *β-catenin* caused enlarged neural tissue and expansion of the neural progenitor pool (Chenn and Walsh, 2002). Downstream of

Wnt signalling, N-myc increases the number of the neural progenitors in the developing neo-cortex (Kuwahara et al., 2010). Moreover, deletion of *N-myc* results in elevated levels of several CKIs, such as *p27* and *p18* (Knoepfler et al., 2002). Shh signalling also has mitogenic activities. Shh induces the expression of a negative regulator of CKI expression, *Bmi1*, and, similarly to Wnt signalling, activates the expression of *N-myc*, which leads to increased expression of *CyclinD1* and *CyclinD2* (Marino, 2005, Salomoni and Calegari, 2010). Interestingly, Fgf signalling regulates cell cycle parameters in the nervous system (Trokovic et al., 2005). Moreover, disturbance of *Fgf2* in the adult nervous system reduces the progenitor pool without affecting the cell cycle length, whereas *Fgf2* in cortical cultures causes lower expression levels of CKIs, higher Cyclin and Cdk levels, and 40% shortening of the G1 (Lukaszewicz et al., 2002, Zheng et al., 2004, Salomoni and Calegari, 2010).

2.1.6.4. Symmetry of cell division and neural fate

Cell division consist of mitosis, the separation of sister chromatids, and cytokinesis, the splitting of cellular components. Near the completion of mitosis, cytokinesis begins. In the neuroepithelial cells, cleavage furrow ingression occurs from a basal-to-apical direction (Fietz and Huttner, 2011). In late cytokinesis, the cleavage furrow has nearly reached the apical surface. At this point, a midbody forms at the apical-most location where daughter cells are still attached through a cytoplasmic bridge (Farkas and Huttner, 2008). The midbody consists of microtubular remnants of the mitotic spindle and a contractile ring (Glotzer, 2005). These structures have been suggested to produce large, prominin1 including particles to the ventricular fluid at the onset of neurogenesis. This might correlate with neural fate by decreasing the surface area of the apical plasma membrane (Dubreuil et al., 2007).

The neuroepithelial cells undergo symmetrical proliferative division, which produces two progenitors (Fig. 4C, Kosodo et al., 2004, Gotz and Huttner, 2005). During neurogenesis, the neuroepithelium derived radial glial cells divide asymmetrically producing one progenitor and one neuron. This asymmetric division is a self-renewal division (Fig. 4C). The basal progenitors divide symmetrically, but they generate two neurons instead of progenitors, and, thus, this division is called symmetrical neurogenic division (Fig. 4C). How cellular constituents are allocated between daughters defines the nature of division. If cellular constituents are distributed equally, cells proliferate symmetrically, and if distribution is unequal, one daughter becomes a progenitor and the other a neuron (Kosodo et al., 2004, Gotz and Huttner, 2005). In *Drosophila melanogaster*, the orientation of the radial cleavage plane (vertical cleavage) has been suggested to result in symmetric, proliferative division and a parallel cleavage plane (horizontal cleavage) in asymmetric division. However, in mammals horizontal cleavage planes are rare (Gotz and Huttner, 2005, Buchman and Tsai, 2007). In highly elongated mammalian progenitors, equal distribution of apical and basal cell fate determinants requires precise bisection of apical and basal constituents. Thus, the orientation of the mitotic spindle has a pivotal role to determine the symmetric versus asymmetric inheritance of cellular components. The inheritance of apical constituents might bias cellular fate towards a more proliferative state (Kosodo et al., 2004). However, recent studies with live imaging techniques have suggested a primary role for heritance of the basal process in the maintenance of the proliferative fate (Konno et al., 2008, Alexandre et al., 2010).

2.1.6.5. Molecular identity of neural progenitors

The molecular regulators of the neural progenitor's decision to self-renew or differentiate include Sox proteins, *Hes* transcription factors, proneural genes, and Notch signalling components.

Sox transcription factors control several key events during neuronal development such as stem cell maintenance, cell fate determination to neuron or glia, and terminal differentiation (Wegner and Stolt, 2005, Bergsland et al., 2011). Stem cell maintenance in neuroectodermal and radial glial cells is regulated by *SoxB1* factors (Wegner and Stolt, 2005). This family of *Sox* genes includes *Sox1*, *Sox2* and *Sox3*. The expression of SoxB1 proteins overlaps and they cooperate in many regions of the developing neuroectoderm. SoxB1 genes are expressed in tissues committed to neuronal fate and expression of *Sox2* is regulated by molecules related to neuronal induction, such as Fgfs and Wnts (Pevny and Placzek, 2005, Takemoto et al., 2006). Overexpression of *SoxB1* factors promotes proliferation and prevents neurogenesis (Wegner and Stolt, 2005). Abolishment of *SoxB1* expression causes premature neuronal differentiation (Graham et al., 2003). SoxB1 factors inhibit the function of proneural genes, which in turn abolish SoxB1 activity. For suppression of SoxB1 function, the proneural genes cooperate with SoxB2 transcription factors. The SoxB2 family includes *Sox21* and *Sox14*, which bind similar target sequences with *SoxB1* factors causing repression rather than activation of transcription (Uchikawa et al., 1999, Sandberg et al., 2005). *Sox14* is expressed in the postmitotic precursors whereas *Sox21* is expressed in neural progenitors in an overlapping manner with *SoxB1* factors (Sandberg et al., 2005). The balance in the expression of *SoxB1* and *SoxB2* affects the decision whether a cell proliferates or differentiates (Wegner and Stolt, 2005). Expression of *SoxC* factors (*Sox4* and *Sox11*) is also promoted by proneural factors, and their activation regulates maturation of immature neurons (Bergsland et al., 2006, Wegner, 2011).

Hes genes encode basic helix-loop-helix transcriptional repressors. *Hes1*, *Hes3* and *Hes5* are expressed in neural progenitors during embryogenesis (Kageyama et al., 2008a). The neuroectodermal cells express *Hes1* and *Hes3* (Hatakeyama et al., 2004). In the beginning of neurogenesis, expression of *Hes3* is reduced and *Hes5* induced (Hatakeyama et al., 2004). Deletion of *Hes* genes causes an upregulation of proneural genes, premature neurogenesis, and a reduction of the stem cell pool (Hatakeyama et al., 2004). In contrast, missexpression of these genes results in an enhanced maintenance of the neural progenitors and reduced neural differentiation (Ohtsuka et al., 2001). *Hes* genes are not required for the initial development of neural progenitors, but are needed to maintain the neural progenitor pool (Kageyama et al., 2008a). Later, *Hes* genes further inhibit the neural fate and promote gliogenesis.

Proneural genes encode basic helix-loop-helix transcription factors, which promote differentiation of neuronal progenitors (Bertrand et al., 2002, Ross et al., 2003). This group of genes includes *Neurogenin 1* (*Ngn1*), *Ngn2*, *Ascl1* (*Mash1*) and *Math1*. The sustained expression of proneural genes prevents proliferation by counteracting the function of SoxB1 and Hes proteins. The proneural genes promote *Hes6* expression, which inhibits Hes1 function at a post-transcriptional level (Bae et al., 2000). *Hes6* expression further accelerates *Ascl1* expression, thus, resulting in the establishment of the neuronal fate. The proneural genes are able to negatively regulate their own activity in neighbouring cells through lateral inhibition: they activate Notch ligand expression, which stimulates Notch signalling and inhibits neuronal fate in cells adjacent to the Notch ligand expressing cell (details below). Thus, proneural genes inhibit

progenitor fate and promote the expression of differentiated neuron markers (Bertrand et al., 2002). Ectopic expression of the proneural genes induces premature cell cycle exit and neurogenesis, and simultaneous deletion of *Ngn2* and *Ascl1* leads to expansion of the progenitor pool (Ross et al., 2003). Recently, it has been shown that *Ascl1* directly regulates genes involved in specification of the neural progenitor, cell cycle progression, terminal differentiation of neurons and neurite outgrowth (Castro et al., 2011). Proneural gene expression indicates a commitment to the neuronal lineage and, thus, inhibits gliogenesis by interacting with Jak-Stat signalling (Vetter, 2001, Guillemot, 2007).

In addition to promoting general post-mitotic neuronal differentiation, specific proneural genes are associated with specification of certain neuronal populations (Guillemot, 2007). In the spinal cord, *Math1*, *Ngn1* and *Ascl1* induce differentiation of distinct interneuron subtypes, and *Ngns* specify the motoneuron identity (Bertrand et al., 2002, Helms et al., 2005, Guillemot, 2007). *Ngn2* promotes the identity of sensory neurons in the peripheral nervous system, whereas *Ascl1* specifies noradrenergic lineages in the peripheral nervous system, as well as the LC neurons in the brain by inducing *Phox2a* and *Phox2b* expression (Bertrand et al., 2002). In the telencephalon, *Ascl1* expression is associated with GABAergic and Ngns with glutamatergic fate (Ross et al., 2003, Guillemot, 2007). In the hindbrain, *Ascl1* promotes serotonergic differentiation. In the ventral midbrain, *Ngn2* induces the differentiation of the dopaminergic neurons (Andersson et al., 2006a, Kele et al., 2006), whereas *Ascl1* is required for specification of different GABAergic subpopulations (Peltopuro et al., 2010). Inactivation of *Ascl1* results in a complete loss of dorsal midbrain GABAergic neurons in m1-m2. In contrast, neurogenesis of m3-m5 derived GABAergic neurons is delayed but not abolished in the *Ascl1^{null}* mutants (Peltopuro et al., 2010) resembling delayed neurogenesis of the dopaminergic neurons in *Ngn2* mutants (Kele et al., 2006). Furthermore, *Ascl1* is not needed for specification of the DA neuron associated VTA/SNpr GABAergic neurons in the ventral midbrain (Peltopuro et al., 2010).

2.1.6.6. Notch oscillation and neurogenesis

Notch signalling is activated by transmembrane ligands, such as Delta-like1 (Dll1), which are expressed by a neighbouring cell (Kageyama et al., 2008b). These ligands bind to the transmembrane protein Notch. The binding releases the intracellular domain of Notch (NICD), which translocates into the nucleus. There NICD forms a complex with cofactor RBP-J causing a conformational change from a repressor to an activator. This activator complex induces expression of *Hes1* and *Hes5*.

Classic view of lateral inhibition. *Hes* genes, the proneural genes, and Notch ligands are expressed in salt-and-pepper patterns in the neural progenitors. Some of the progenitor cells seem to express these genes at high levels and others nearly lack expression. This is explained by lateral inhibition. In the classic view of lateral inhibition, all neuroepithelial cells initially express the proneural genes and Notch ligands at equal levels (Kageyama et al., 2008b). Stochastic variation results in slightly higher Notch ligand levels in some of the cells and, thus, induction of Notch signalling and *Hes* expression in adjacent cells. This inhibits expression of the proneural genes and Notch ligands. Therefore, the cells that expressed Notch ligands at higher levels are not exposed to high levels of *Hes* expression and are able to express the proneural genes, Notch ligands and, finally, differentiate into neurons. The salt-and-pepper pattern of proneural genes and Notch ligands has been suggested to reveal the beginning of neurogenesis (Kageyama et al., 2008b).

Notch oscillation. In the neural progenitors, variable levels of *Hes1* appear already at E7.5 (Kageyama et al., 2008b). The proneural genes, *Ngn2* and *Ascl11*, and the Notch ligand *Dll1* are expressed only slightly later, at E8-E8.5. Thus, initiation of *Hes1* expression appears independent of Notch signalling, but becomes Notch dependent after initiation of *Dll1* expression. Ascl1 and Ngn2 are able to bind enhancer regions of *Dll1* and, thus, promote *Dll1* expression. Real-time imaging analysis shows that *Hes1* expression oscillates in neural progenitors with 2-3 hour periods (Shimojo et al., 2008). Moreover, the expression of *Ngn2* and *Dll1* appear to oscillate in a phase opposite to *Hes1* oscillation. *Hes1* oscillation seems to regulate *Ngn2* oscillation, and *Ngn2* is further regulating *Dll1* expression. In the postmitotic neurons, the expression of *Hes1* is repressed probably through a Notch inhibitor, *Numb* or *Nato3,* resulting in elevation of *Ngn2* and *Dll1* expression (Johnson, 2003, Ono et al., 2010). Oscillation of *Ngn2* does not induce neurogenesis, because cells might require longer exposure time to induce neuronal differentiation, and *Hes1* expression between *Ngn2* waves inhibits neurogenesis (Shimojo et al., 2008). However, accumulation of proneural factors or neurogenic fate determinants during *Hes1/proneural* gene oscillation, and their influence on neural commitment remains to be elucidated.

Revised view of lateral inhibition. These findings suggest a revised view of lateral inhibition (Kageyama et al., 2008b). *Hes1* oscillation regulates *proneural gene/Dll1* oscillation in an opposite phase, and Notch-mediated lateral inhibition occurs simultaneously in the neuroectodermal cells before neurogenesis. The oscillation of proneural genes does not strictly predict neuronal differentiation, but is needed for activation of intercellular Notch signalling. This maintains *Hes1* oscillation and, thus, is required for maintenance of the progenitor cells. Upon cell cycle exit the postmitotic precursors express the proneural genes and *Dll1* in a sustained manner, which then activates Notch signalling and *Hes* expression in adjacent cells and promotes the maintenance of the progenitor pool (Kageyama et al., 2008b).

Regulation of Hes oscillation. Hes1 appears to bind its own promoter in order to repress its own expression (Shimojo et al., 2011). This negative feedback mechanism causes rapid downregulation of *Hes1* mRNA and Hes1 protein, which are unstable. These feedback actions initiate autonomously of the oscillatory expression of *Hes1* (Hirata et al., 2002, Shimojo et al., 2011). Furthermore, Hes1 oscillation is regulated by oscillatory Jak-Stat signalling (Yoshiura et al., 2007). Activated Jak/Stat signalling activates target gene (such as *Socs3)* expression. Soc3 through, negative a feedback loop, inhibits phosphorylation of Stat3 that further induces the oscillation of phosphorylated Stat3 and expression of *Soc3* (Yoshiura et al., 2007). Inhibition of this oscillation causes a disruption in Hes1 oscillation (Kageyama et al., 2008b, Shimojo et al., 2008).

2.2. FGF signalling

Fgf signalling plays an important role in multiple processes and many tissues during development. These processes include cell proliferation, survival, differentiation, and fate determination. Since Fgf signalling has a primary role in wide variety of biological functions, it is one of the most studied signalling pathways (Itoh and Ornitz, 2008). In early embryos, Fgf signalling regulates early patterning, mesoderm specification, axis formation, cell movements, and neural induction (Thisse and Thisse, 2005, Dorey and Amaya, 2010). Later, it has functions,

for example, in the patterning of several brain regions, in the induction and morphogenesis of the limbs, and in the formation of bone structures.

In the limb development, Fgf8 initiates limb bud development by inducing the expression of *Fgf10*, which mutually activates *Fgf8* in the epithelial cells. These *Fgf8* expressing ectodermal cells form a signalling centre, the apical ectodermal ridge, in the distal tip of the limb bud. This signalling centre maintains cell proliferation in the underlying mesenchymal cells. Moreover, Fgf8, together with Fgf2 and Fgf4, induces and maintains the expression of *Shh* in mesenchymal cells in the posterior-proximal part of the limb bud. These *Shh* expressing cells form another signalling centre called a zone of polarizing activity. These two signalling centres orchestrate the outgrowth and patterning of the limbs (Gilbert, 2003, Thisse and Thisse, 2005). Fgf signalling is also required for bone formation. Fgf18 is needed for differentiating osteoblasts in calvarial bones and for development of the long bones (Ohbayashi et al., 2002). Furthermore, many skeletal malformations in humans are associated with mutations in the Fgf signalling pathway. The point mutation in the *Fgfr3* gene is the most common genetic cause of dwarfism. Activating mutations in *FGFR1* or *FGFR2* causes skeletal dysplasias, in which one or two cranial sutures fuses prematurely (Miraoui and Marie, 2010). Since many skeletal disorders are caused by sustained Fgf signalling, the systems or signalling pathways that antagonise or crosstalk (such as Wnt, Egf, and PDGF signalling pathways) with Fgf signalling may offer therapeutic potential for these skeletal disorders (Miraoui and Marie, 2010). In many tissues Fgf signalling operates through epithelium-mesenchyme interaction. In the branching morphogenesis of the lung, mesenchymal *FGF9* through epithelial *FGFR2b* activates and regulates the expression and function of *FGF10* in the bronchial mesenchyme. Thus, mesenchymal activation is required to induce branching morphogenesis in the lung epithelium (Warburton et al., 2008). Similarly, the interaction of mesenchymal Fgf10 and epithelial Fgfr2b controls morphogenesis in the developing tooth, palate and calvarial bones (Veistinen et al., 2009). Disruption of this interplay by inactivating either *Fgfr2b* or *Fgf10* causes decreased proliferation in developing tooth or even failure of molar tooth formation.

In addition to developmental roles, Fgf signalling is needed for tissue repair and the regulation of nutrition and energy metabolism in adults (Itoh, 2007, Beenken and Mohammadi, 2009, Do et al., 2012). Misexpression of some Fgf signalling components is involved in the progression of several cancers (Beenken and Mohammadi, 2009, Turner and Grose, 2010).

2.2.1. Fgfs and Fgf receptors

Fgf ligands. Fibroblast growth factors (Fgfs) are a large group of polypeptide growth factors that have been conserved during the evolution of metazoans (Itoh and Ornitz, 2011). During evolution, Fgf-like genes were expanded in two phases. In the first phase ancestors of Fgf subfamilies were generated and in the second phase the subfamilies duplicated to contain several members (Itoh and Ornitz, 2008). Thus, two *Fgf -like* genes are described in nematode *Caenorhabditis elegans (C. elegans),* six in ascidian *Ciona intestinalis (Ci. intestinalis)* and sixteen in zebrafish. The mammalian Fgf protein family contains 22 members, which are recognised by an Fgf-specific conserved core about 120 amino acids (Itoh, 2007, Sunmonu et al., 2011b). This conserved domain is required for receptor binding (Sunmonu et al., 2011b). Fgfs can be divided into seven subfamilies based on sequence homology, genomic location and function (Itoh and Ornitz, 2008, Itoh and Ornitz, 2011). Most of Fgf subfamilies mediate signalling through Fgf receptors (Fgfrs) and are called canonical subfamilies. These are the

Fgf1/2 subfamily, Fgf4/5/6 subfamily, Fgf3/7/10/22 subfamily, Fgf9/16/20 subfamily and Fgf8/17/18 subfamily. Intracellular Fgf11/12/13/14 subfamily proteins act inside cells without binding to cell surface receptors. Hormone-like Fgf15/21/23 subfamily proteins act in an Fgfr-dependent-manner, although binding to receptors requires Klotho -cofactor activity, and the binding affinity appears to be relatively low. The mouse *Fgf15* is orthologous to human *FGF19*. Most secreted hormone-like and canonical Fgfs are released from cells by the conventional amino terminal signal peptide cleavage mechanism (Itoh and Ornitz, 2008). However, Fgf1/2/9/16 and 20 are also secreted molecules though they lack a secretion signal sequence at their N-terminus.

Fgf receptors. Four different *Fgfr* genes (*Fgfr1-Fgfr4*) have been characterized from human and mouse, whereas just one Fgfr has been identified from *C. elegans* and *Ci. intestinalis* (Itoh, 2007). These encode cell-surface tyrosine-kinase receptors containing an extracellular ligand-binging domain, a transmembrane domain and an intracellular tyrosine kinase domain. The extracellular domain includes three immunoglobulin-like domains (I, II and III, Fig.5). The acid box and HSPG binding site are located between the immunoglobulin-like domains I and II (Guillemot and Zimmer, 2011). Fgfr1, Fgfr2, and Fgfr3 have two alternative splice variants in the domain III, IIIb and IIIc isoforms, which are expressed in a tissue-specific manner (see also Fgfs in patterning of IsO, page 13). Domain III is required for the binding specificity of Fgf ligands, and, thus, IIIb and IIIc isoforms have very different roles in Fgfr function (Ornitz and Itoh, 2001). Alternative splicing of Fgfs and Fgfrs affects receptor binding affinities and, thus, increases a variety of biological activities regulated by Fgf signalling (Itoh, 2007). The acid box is needed for cell adhesion activities. When Fgf ligand binds to Fgfr, two Fgfr monomers dimerise and the intracellular tyrosine-kinase domains cross-phosphorylate each other to activate downstream signalling pathways. The protein encoded by *Fgfr- related* gene (*FgfrL1*) a lacks tyrosine binding domain although it has Fgfr binding activity (Mason, 2007). Hence, FgfrL1 likely acts as an antagonist than as an inducer of Fgf signalling.

Heparan sulphate proteoglycans. Canonical Fgfs have a binding site for co-factor heparin or heparan sulphate proteoglycan (HSPG) and together with Fgfr they form an Fgf-Fgfr-heparin complex (Fig.5, Sunmonu et al., 2011b). HSPGs stabilize the interaction between Fgf ligand and Fgfr. In addition to contributing to the formation of the Fgf-Fgfr-HSPG complex, HSPGs can affect binding specificity and restrict Fgf diffusion and protein degradation (Mason, 2007, Guillemot and Zimmer, 2011). Hormone-like Fgfs bind to heparin with low affinity, allowing their distribution and function as hormones (Goetz et al., 2007, Sunmonu et al., 2011b). HSPGs enhance autophosphorylation of Fgfrs by bringing two subunits required for dimerization near each other and allowing the dimer formation (Ornitz and Itoh, 2001).

2.2.2. Fgf signalling pathways

MAPK/ERK pathway. Fgf signalling can be transduced through several downstream pathways. Through the mitogen-activated-protein kinase/extracellular-signal-regulated kinase (MAPK/Erk) signalling cascade, Fgfs regulate proliferation, migration, differentiation and the
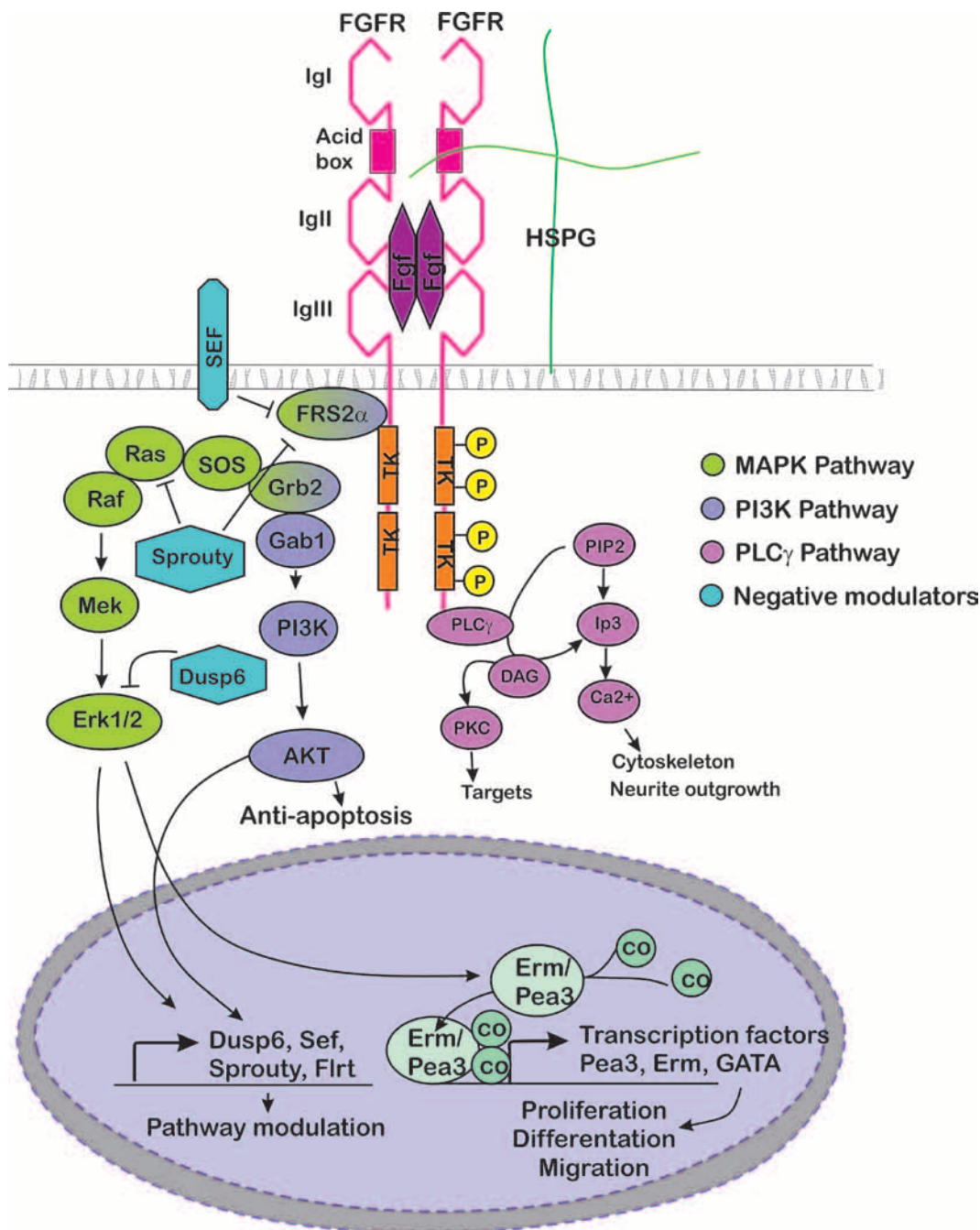
Figure 5. **Fgf signalling.** Fgf signalling is activated when Fgf molecules bind to extracellular, immunoglobulin-like domains (II and III) of Fgf receptor, which causes the dimerization of the receptor molecule. This dimerization induces the cross-phosphorylation of intracellular, tyrosine kinase domains, which further induces downstream signalling through MAPK, PI3K or PLCγ pathways. Heparan sulphate facilitates the formation of the complex between Fgfs and Fgfrs. The acid box of Fgf receptors interacts with adhesion molecules such cadherins. The MAPK pathway induces expression of distinct transcription factors, which regulates cell proliferation, differentiation and migration. In addition, the MAPK pathway regulates the expression of negative modulators of Fgf signalling pathway and, thus, induces a regulative feedback loop. Through the PLCγ pathway Fgf signalling regulates for example cytoskeleton and neurite outgrowth, and the PI3K pathway is involved in anti-apoptotic functions. Fgf Fibroblast growth factor, Fgfr Fibroblast growth factor receptor, HSPG heparan sulphate proteoglycan, Ig immunoglobulin-like domain, TK tyrosine kinase domain, p phosphorylation site, co cofactor. (Thisse and Thisse, 2005, Mason, 2007, Guillemot and Zimmer, 2011).

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expression of feedback regulators (Fig.5, Guillemot and Zimmer, 2011). The MAPK pathway is activated when the Fgfr intracellular domain interacts and activates a membrane-anchored docking protein Frs2α (Thisse and Thisse, 2005). Frs2α activation provides binding sites for a small adaptor molecule Grb2. Grb2 appears to form a complex with a nucleotide exchange factor Sos. Sos activates a small GTPase Ras by catalysing the exchange of guanosine diphosphate (GDP) to guanosine triphosphate (GTP). Ras activation causes phosphorylation of the proto-oncogene serine/threonine protein kinase (Raf), which further induces phosphorylation of mitogen-activated protein kinase kinase (MEK). The next component of the signalling cascade, mitogen activated protein (MAP) kinase or extracellular signal regulated kinase (Erk), is phosphorylated by MEK. This activation releases Erk proteins from surrounding proteins and they are translocated into the nucleus. In the nucleus, they phosphorylate downstream transcription factors such as Pea3 (Etv4) and Erm (Etv5), which together with certain cofactors bind to promoter regions of target genes to activate or repress expression (Tsang and Dawid, 2004).

PLCγ pathway. Activation of the Phospholipase Cγ (PLCγ/Ca^{2+}) pathway stimulates neurite outgrowth and is associated with the modulation of cytoskeleton (Fig.5, Guillemot and Zimmer, 2011). The Src homology 2 (SH2) domain of PLCγ binds the tyrosine residue (Tyr 766) of Fgfr after autophosphorylation (Thisse and Thisse, 2005). Activated PLCγ hydrolyses phosphatidylinositol-4,5-diphospate (PIP2) to inositol-1,4,5-triphosphate (IP3) and diacylglycerol (DAG). IP3 is able to induce Ca^{2+} release from storage, while DAG activates protein kinase C (PKCδ), which is able to phosphorylate Raf and activate the MAPK pathway.

Phosphoinositol-3-kinase pathway. In the phosphoinositol-3-kinase (PI3K) pathway PI3K is activated when Gab1 binds to Frs2 through Grb2 (Fig.5). This induces PI3K to phosphorylate PIP2 to generate phosphatidylinositol-3,4,5-triphospate (PIP3) which induces serine/threonine kinase Akt activation (Katoh and Katoh, 2006). The PI3 kinase/Akt pathway has anti-apoptotic activities in the nervous system.

Through Frs2 and small GTPases Fgf signalling modulates the cytoskeleton and stimulates neurite outgrowth (Guillemot and Zimmer, 2011). *Erm*, *Pea3*, *Fos, Jun* and *GATA* factors are transcriptional activator genes, whose expression is induced by the Erk/MAPK pathway. These transcriptional effectors regulate cell proliferation, differentiation and migration (Guillemot and Zimmer, 2011). In zebrafish, Canopy family protein, Canopy1, is induced by Fgf signalling and it contributes to positive regulation of the Fgf signalling pathway by interacting with Fgfr1 in the midbrain-r1 region (Hirate and Okamoto, 2006). Several other feedback regulators, such as *Sproutys*, *Sef* and *Mkp3*, are activated through the Fgf signalling cascade.

2.2.3. Feedback modulators of Fgf signalling

Fgf signalling regulates several transcriptional target genes. Some of them participate in feedback regulation or modulation of the Fgf signalling pathway. Most of these feedback regulators act as negative regulators (Fig.5). These include the cytosolic proteins Sprouty and Mkp3, as well as the transmembrane protein Sef (Mason, 2007). These regulatory inhibitors control tightly this signalling cascade and, thus, allow primary role of Fgfs in large variety of developmental processes (Thisse and Thisse, 2005).

Sproutys. Four *Sprouty* genes are found in vertebrates and three of them, *Sprouty1*, *Sprouty2* and *Sprouty4* are expressed in the midbrain-hindbrain territory in an overlapping manner with

Fgf8 (Echevarria et al., 2005a). The Sprouty proteins are negative feedback modulators of the Ras/MAPK pathway without affecting other Fgf downstream signalling pathways (Fig. 5). The Sprouty proteins act redundantly and regulate the Ras/MAPK pathway between Fgfr tyrosine kinase phosphorylation and Ras activation (Fig.5). Misexpression of *Sprouty2* in chick embryos caused decreased activation of the Erk pathway and fate change from hindbrain primordia to midbrain primordia (Suzuki-Hirano et al., 2010). During cerebellar development, strong Erk activation caused by Fgf8 is needed for induction of the cerebellar fate (Matsumoto et al., 2004). This upregulation of Erk, however, has to be downregulated by Sprouty2 to achieve the cerebellar fate (Suzuki-Hirano et al., 2010).

Dusp6. Erk activity is also negatively regulated by MAP kinase phosphatases (MKPs, Fig.5)) (Echevarria et al., 2005a). *Mkp3* (also known as *Dusp6*) is expressed in the midbrain-hindbrain region. Moreover, expression of *Dusp6* and *Fgf8* localizes in several positions in the developing neural tube, especially in secondary organizer regions such as the ANR and the IsO. Fgf8 soaked beads induced *Dusp6* expression in ectopic locations of the neural tube indicating direct regulation of *Dusp6* by Fgf8. This regulation is mediated through the PI3K pathway (Echevarria et al., 2005b). However, *Dusp6^{null}* mutants lack neuronal changes during embryonic development (Li et al., 2007). Thus, Dusp6 is not specifically regulating a certain Fgfr, or is not a negative modulator of all Fgf signals.

Sef. The transmembrane protein Sef (similar expression to Fgfs) is conserved among vertebrates and is similarly expressed with *Sproutys* and *Dusp6* in the midbrain-anterior hindbrain (Echevarria et al., 2005a). Sef inhibits tyrosine phosphorylation of Fgfr1 and Fgfr2, but not Fgfr3 (Tsang et al., 2002). The mechanism, how Sef regulates the signalling activity, is not fully understood. Several studies suggest that Sef functions by inhibiting receptors or inactivating the cofactor Frs2 before Ras activation (Fig.5; Kovalenko et al., 2003, Kovalenko et al., 2006). Signal modulators Sef and Sproutys, especially Sprouty2, cooperate in the regulation of Fgf signalling, since simultaneous abolishment of these genes causes upregulation of *Gbx2*, a downstream target of *Fgf8* (Lin et al., 2005).

2.2.4. Fgf and Fgfr expression is required in the development of the midbrain and anterior hindbrain

Fgfs. *Fgf8*, *Fgf17*, *Fgf18* and *Fgf15* are expressed in the midbrain-hindbrain region (Fig. 6). In the midbrain-hindbrain territory, *Fgf8* expression is initiated at the Otx2-Gbx2 border at the 3-5-somite stage (Crossley and Martin, 1995). *Fgf8* is expressed in broad domain at E8.5 but gets restricted to a narrow stripe in the *Gbx2*-expressing hindbrain side of the MHB at E9.5. *Fgf17* and *Fgf18* expression begins slightly after *Fgf8* expression. *Fgf17* expression appears to be weaker than *Fgf8* at E8.5, but after E11.5, *Fgf17* expression continues stronger than *Fgf8*, suggesting a role in later development (Xu et al., 1999). Fgf17 expression overlaps with *Fgf8*, but forms broader pattern on both sides of the midbrain-hindbrain border. During early development Fgf8 is needed for gastrulation, induction of caudal fate in the neural tube, and establishing left-right asymmetry in the primitive streak (reviewed in Sunmonu et al., 2011b). Thus, embryos lacking Fgf8 die at E8.5 (Sun et al., 1999). Conditional inactivation experiments have revealed that Fgf8 has a crucial role in several regions where it is expressed during neurogenesis (Chi et al., 2003, Hebert, 2011). Moreover, midbrain-anterior hindbrain specific inactivation of *Fgf8* (*Fgf8^{cko}*) causes large deletions throughout the midbrain and anterior hindbrain territory including the loss of both dorsal structures, such as the tectum and the cerebellum, and part of ventral regions (Chi et al., 2003). Important brain nuclei located in the region such as the SN, the VTA, the LC, and the III and the IV cranial ganglia are also abol-

ished. The maintenance of the MHB specific genes fails in this $Fgf8^{cko}$ mutants. The failure in MHB specification leads to ectopic apoptosis especially in the dorsal regions.

Fgf15 is expressed in the midbrain and anterior hindbrain region, but is not expressed in a narrow boundary cell population at the MHB (Fig. 6A, Gimeno et al., 2003, Trokovic et al., 2005). $Fgf15^{null}$ mutant mice are viable (Wright et al., 2004), but during the development of the neocortex *Fgf15* inhibits proliferation and induces neural differentiation (Borello et al., 2008). In the absence of *Fgf15*, progenitors of the dorso-lateral midbrain are not differentiating. The expression of genes promoting proliferation, such as *Id1*, *Id3* and *Hes5*, is increased, whereas proneural factors *Ascl1*, *Ngn1* and *Ngn2* are downregulated (Fischer et al., 2011). Thus, *Fgf15* is needed for cell cycle exit and proper neurogenesis in the dorsal midbrain.

Fgf receptors. Downstream effects of Fgf8 are mediated through Fgfrs. *Fgfr1* and *Fgfr2* are expressed in the head-folds already at E7.5 (Trokovic et al., 2005). From E8.5 to E12.5, *Fgfr1* is expressed evenly throughout the region (Fig. 6B, Blak et al., 2005, Trokovic et al., 2005). In contrast, *Fgfr2* and *Fgfr3* show more dynamic expression patterns (Fig. 6; Blak et al., 2005, Trokovic et al., 2005). At E8.5 *Fgfr2* is expressed in the anterior midbrain, but is not expressed in the MHB or r1. By E9.5, the ventral expression of *Fgfr2* is restricted to *Fgf8* zone, but dorsally *Fgfr2* expression does not reach the *Fgf8* expression domain. Later, the dorsal expression of *Fgfr2* approaches the *Fgf8* domain, and expression spreads also in the
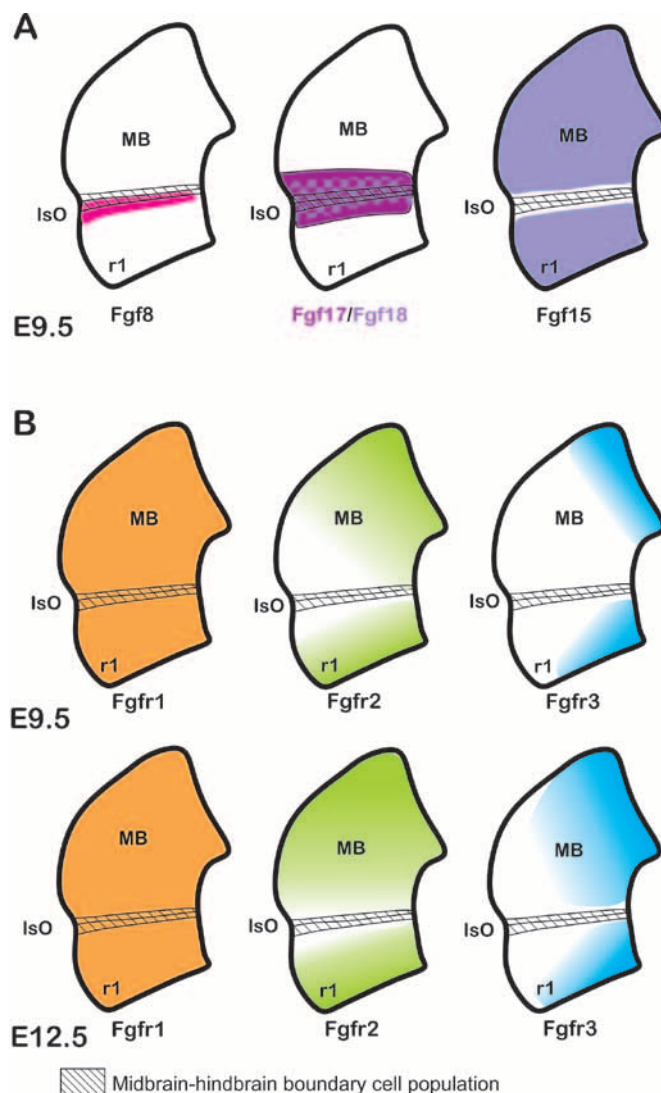

Figure 6. **Expression of Fgfs and Fgf receptors in the midbrain-anterior hindbrain region.** *Fgf8*, *Fgf17* and *Fgf18* are expressed in the midbrain-hindbrain boundary **(A)**. First these signalling molecules are expressed in broader domains, but are soon restricted to the boundary region. *Fgf8* is expressed in the most anterior hindbrain. *Fgf17* and *Fgf18* are expressed on both sides of the boundary. *Fgf15* is expressed throughout the midbrain and rhombomere1 but is downregulated/lacking from the midbrain-hindbrain border **(A)**. Three Fgf receptors, *Fgfr1*, *Fgfr2* and *Fgfr3*, are expressed in the developing brain **(B)**. *Fgfr1* is expressed throughout the region during early neurogenesis. *Fgfr2* and *Fgfr3* show more restricted expression patterns. Neither of these is expressed in the specific midbrain-hindbrain boundary cell population. They are expressed in the anterior midbrain and caudal rhombomere 1, but expression decreases as a gradient near the border of the midbrain and hindbrain. The dashed area marks the specific midbrain-hindbrain boundary cell population. MB midbrain, r1 rhombomere1, IsO isthmic organizer. (Blak et al., 2005, Trokovic et al., 2005, Partanen, 2007)

postmitotic cell layers. At E8.5, *Fgfr3* is not expressed in the midbrain or r1. At E9.5-E10.5, *Fgfr3* is expressed in the anterior midbrain; in the ventral r1 the expression is restricted to the *Fgf8* expression domain. *Fgfr3* expression is lacking from MHB at E8.5-E12.5. Later, *Fgfr3* expression expands in a broader area in the midbrain and r1 concentrating in the ventral regions. However, the expression of both *Fgfr2* and *Fgfr3* increases as a gradient towards the diencephalon and r2. *Fgfr4* is not expressed in the CNS during early development. Thus, *Fgfr1* is expressed throughout the midbrain and anterior hindbrain, whereas *Fgfr2* and *Fgfr3* have more restricted expression domains. Therefore, Fgfr1 is considered to be the primary transducer of Fgf signals in the midbrain and anterior hindbrain during early development.

The early expression of *Fgfr1* and *Fgfr2* suggests an important role for these molecules during early development. Indeed, null mutants for *Fgfr1* and *Fgfr2* die before E9.5 (Dorey and Amaya, 2010). *Fgfr1^{null}* mutants have defects in cell movement during gastrulation and paraxial mesoderm is lost. *Fgfr2^{null}* mutants have failures in visceral endoderm differentiation and maintenance of inner cell mass. However, *Fgfr3^{null}* mutant mice are viable and they have some skeletal abnormalities, but only minor changes in the CNS. Thus, conditional-mutagenesis approach is needed to study function of these genes at later stages.

Inactivation of Fgfr1 in the midbrain-rhombomere1 region. Midbrain-anterior hindbrain specific inactivation of *Fgfr1* by En1-Cre (*Fgfr1^{cko}*) results in downregulation of *Fgfr1* expression from E8.5 onwards being totally lost by E9.5. These *Fgfr1^{cko}* mutants survive until adulthood, but the dorsal structures, such as the vermis of the cerebellum and the inferior colliculi of the midbrain, are lost and direct Fgf downstream targets, such as *Erm*, *Pea3* and *Sproutys*, are downregulated in the border of the midbrain and hindbrain (Trokovic et al., 2003). The ventral regions remain mainly intact and the MHB specific gene expression show relatively minor disruptions compared to the *Fgf8^{cko}* mutants. This finding suggests that besides Fgfr1 other Fgf receptors, such as Fgfr2 and Fgfr3, may mediate Fgf signals in the midbrain and anterior hindbrain region.

2.2.5. Fgfr1 regulates a boundary cell population at the midbrain-hindbrain border

The Boundary cells. The cells at compartment boundaries often display specific characteristics. They have distinct adhesive properties, the boundary cells proliferate slowly and prevent neurogenesis (Kiecker and Lumsden, 2005, Kiecker and Lumsden, 2012). Adjacent compartments may express different cell adhesion molecules, which ensure cell segregation between different compartments. In the hindbrain, various *Ephrin* receptors (*Eph*) are expressed in odd-numbered rhombomeres, whereas *Ephrins*, the ligands, are expressed in even-numbered rhombomeres (Kiecker and Lumsden, 2005). The contact-mediated repulsion between these molecules causes cell segregation between different rhombomeres. Similarly, different cadherins maintain segregation between subdivisions of the brain when they are separately expressed in different compartments (Kiecker and Lumsden, 2005). Other boundary regions, such as the MHB, the floor plate and the roof plate, express *Hes1* in a sustained manner (Baek et al., 2006, Kageyama et al., 2009). This causes repression of neurogenesis. Deletion of *Hes1* from boundary regions causes ectopic expression of *Hes3*, *Hes5* and proneural genes, as well as increased neurogenesis (Baek et al., 2006). Hes1 is able to regulate both positive and negative cell cycle regulators and, thus, the role of Hes1 in cell cycle progression is not fully understood (Kageyama et al., 2008b, Salomoni and Calegari, 2010). However, forced expression of *Hes1* inhibits *CyclinD1* and *CyclinD2*, and delays G1 progression (Baek et al., 2006). The

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boundaries express *Hes1* in a sustained manner and, thus, may proliferate more slowly by a Hes-related mechanism (Kageyama et al., 2008b).

The midbrain-hindbrain specific boundary cell population. Analysis of the *Fgfr1^{cko}* embryos lead to the discovery of a specific midbrain-hindbrain boundary cell population. In the *Fgfr1^{cko}* mutants, the cells in the midbrain-hinbrain border are the most abolished, and the isthmic constriction is lost. The expression borders of Fgf8, Wnt1, Otx2 and Gbx2 are inter- mingled, and cells expressing these genes mix with non-expressing cells, indicating the loss of coherent architecture of the IsO (Trokovic et al., 2003). The cells in the midbrain-hindbrain border express negative cell cycle regulators, such as p21 and Jumonji, and, thus, these specif- ic boundary cells proliferate slower than their surroundings. In *Fgfr1^{cko}* embryos, the specific midbrain-hindbrain boundary cells are lost. These specific boundary cells are located on both sides of the midbrain-hindbrain border in embryos and adults (Trokovic et al., 2005, Kala et al., 2008). Fate-map analysis with a boundary cell-specific Cre- mouse line revealed the loca- tion of boundary derived cells near the midbrain-pons border also in the adult brain, suggest- ing limited cell mixing between the midbrain and the hindbrain. Indeed, it was recently shown that the midbrain-hindbrain border is a true compartment boundary that prevents cell mixing between midbrain and hindbrain compartments (Langenberg and Brand, 2005, Sunmonu et al., 2011a). Partial deletion of *Fgf8* caused *Gbx2* expressing cells to cross over the midbrain- hindbrain border and, thus, *Fgf8* appears to regulate cell sorting between the midbrain and the hindbrain (Sunmonu et al., 2011a). The cell adhesion molecule *Cadherin22* (*Cdh22*), known as *PB-cadherin*, is normally expressed in these midbrain-hindbrain specific boundary cells (Trokovic et al., 2003, Kala et al., 2008). However, the expression of *Cdh22* is downregulated in *Fgfr1^{cko}* mutants (Trokovic et al., 2003). This downregulation of *Cdh22* might lead to a loss of adhesive characters of a coherent compartment boundary allowing mixing of cells across the midbrain-hindbrain border.

2.3. Cell adhesion in the brain

2.3.1. Role of cell adhesion in the developing brain

In the developing nervous system, cell adhesion is needed for multiple processes: the structur- al integrity of certain neuronal populations or subdivisions, the establishment of apico-basal polarity in the neuroepithelial cells, the regulation of cell movements during cell sorting, cell rearrangements and cell migration, the establishment and maintenance of compartment boundaries, neurite outgrowth and pathfinding, synaptogenesis, as well as modulation of neu- ronal circuits within the CNS (Redies, 2000, Tepass et al., 2000, Takeichi, 2007, Suzuki and Takeichi, 2008).

Several families of cell adhesion molecules (CAM) mediate these adhesive functions during neuronal development (Fercakova, 2001). CAMs are usually transmembrane proteins that bind to other CAMs on adjacent cells. They include the immunoglobulin superfamily, cadher- in, integrin, and nectin families. The binding may be homophilic, when CAM binds an identi- cal molecule, or heterophilic, when binding occurs between different molecules (Togashi et al., 2009). CAMs are able to cluster at the cell surface to stabilize adhesion or function as a signalling molecule. This adhesion property is called *cis* adhesion, whereas binding to CAM in the adjacent cell surface is called *trans* adhesion (Redies, 2000).

Immunoglobulin superfamily CAMs are usually transmembrane proteins, which include several immunoglobulin-like (Ig) domains and Fibronectin type III (FNIII) domains in their extracellular part. Neural adhesion molecule, N-Cam, contains five Ig domains and two FNIII repeats, whereas the other member of the family, L1, contains six extracellular Ig domains and five FNIII repeats (Shapiro et al., 2007). Multiple family members might be expressed on the cell surface where they simultaneously regulate adhesive properties. Moreover, soluble forms of IgCAMs are able to modulate adhesive functions (Shapiro et al., 2007). Interestingly, immunoglobulin CAMs appear to cluster through heterophilic binding with Fgfr and this interaction activates Fgf signalling and axonal growth without canonical Fgf ligands (Quarles, 2004). Cadherins also contain five extracellular Ig domains and appear to bind by homophilic interactions (Suzuki and Takeichi, 2008). The cadherin superfamily includes over 100 members and they are involved in multiple processes during nervous system development (see below).

Other cell adhesion molecules in the brain. Integrins are mediators between the extracellular environment and the cytoplasm. They are cell surface glycoproteins, and consist of α and β subunits which form heterodimers (Shapiro et al., 2007). They bind to other cell surface or extracellular matrix molecules such as Laminin or Fibronectin in a heterophilic manner. In the CNS, Integrins are expressed in the neural progenitors, mature neurons, glia and meningeal cells, and are involved in neuronal migration along radial glial fibers, cellular integrity near the pial surface, and synaptogenesis (Milner and Campbell, 2002). Nectins are Immunoglobulin-like CAMs, which are able to bind either in a homophilic or heterophilic manner, heterophilic interaction resulting in a stronger binding than homophilic interaction (Togashi et al., 2009). They are able to stabilize cell-to-cell contacts by forming interactions with cadherins (Tepass et al., 2000, Takeichi, 2007). This cooperation appears in synaptic junctions where neurotransmitter releasing zones are flanked with synaptic adherent junctions. These junctions are formed by a heterophilic interaction between Nectin1 and Nectin3, and interactions with cadherins (Togashi et al., 2009). Cadherins alone are not able to achieve such connections (Takeichi, 2007).

2.3.2. Cadherins in brain development

Cadherins are transmembrane proteins, which are recognized by extracellular (EC), Ig cadherin repeats. Many cadherins are linked to the actin-cytoskeleton through their intracellular domain. Linking to the actin-cytoskeleton might not be essential for cell adhesion but is required for cell arrangements, shape and polarity changes as a driving force (Gumbiner, 2005). Most cadherins probably function without linkage to the actin-cytoskeleton at least in the nervous system (Gumbiner, 2005). The cadherin superfamily can be divided into subgroups: protocadherins, atypical cadherins, desmosomal cadherins and classical cadherins (Takeichi, 2007). The protocadherins have six to seven EC domains, and intracellular domain is not conserved among members of this family (Halbleib and Nelson, 2006). They have relatively weak adhesive properties and the mechanism how they mediate cell adhesion remains largely unknown. However, they might be able to induce intracellular signalling cascades as a response to external interactions. The protocadherins are primarily expressed in the neuronal tissues. This family contains more than 60 members and most of them can be clustered into three groups: *Pcdh-α, Pcdh-β*, and *Pcdh-γ* (Halbleib and Nelson, 2006). In neural development, the proto-

cadherins are required for regulation of synaptic plasticity and modulation of neuronal circuits. The atypical cadherins contain Dashsous, Fat and Flamingo. These cadherins have large structural differences compared to the classical cadherins: Dashsous and Fat has 27 and 34 EC domains, respectively, whereas Flamingo has a seven-pass transmembrane domain (Halbleib and Nelson, 2006). These cadherins are involved in tissue polarity maintenance, controlling proliferation, and regulation of cell movements. The desmosomal cadherins usually have five EC domains, and a conserved intracellular domain. They are localized at the desmosomes (Tepass et al., 2000). The classical cadherins contain 23 members and they are expressed throughout the nervous system (see below).

2.3.2.1. Homophilic adhesion is typical for classical cadherins

Classical cadherins are cell surface glycoproteins that interact by Ca^{2+}-dependent homophilic cell-to-cell adhesion (Halbleib and Nelson, 2006). Generally, the classical cadherin expression concentrates in the adherence junctions of neuroectodermal cells. This apical expression is required for apico-basal polarity. The classical cadherins contain five extracellular Ig domains, a transmembrane domain and an intracellular catenin-binding domain (Halbleib and Nelson, 2006). The EC domains are connected through Ca^{2+} bridges, and form *cis* or *trans* interactions with other CAMs. The intracellular domains connect cadherins to the actin cytoskeleton through β-catenin and α-catenin linkage. The classical cadherins can further be divided into type I (E-Cdh, N-Cdh, P-Cdh, R-Cdh, Cdh15) and type II (Cdh5-Cdh12, Cdh18, Cdh19, Cdh22) cadherins (Nollet et al., 2000). The type I cadherins have a highly conserved HAV (Histidine-Alanine-Valine) tripeptide in their first EC and, thus, they appear to form strong homophilic cell-cell contacts (Halbleib and Nelson, 2006). This tripeptide promoting binding affinity and specificity is lacking from type II cadherins. Moreover, type I cadherins have one tryptophan residue in the EC1 domain anchoring cadherin to the hydrophobic pocket of its binding partner, whereas the type II cadherins contain two tryptophan residues, and thus appear to have a wider binding pocket (Patel et al., 2006). This diminishes adhesion affinity and specificity in type II cadherins. For example, the cells expressing E- or N-Cdh are aggregating more efficiently than cells expressing Cdh7 or Cdh11 (Chu et al., 2006). The other EC domains are related to *cis* binding activities. For example, interaction between N-Cdh and Fgfr is mediated through EC4 (Halbleib and Nelson, 2006).

The classical cadherins in cell segregation. The homophilic binding specificity is the basis of cadherin function for cell segregation. Six different cadherins are expressed in different distributions within the lateral motor column causing cells to segregate into different motor pools based on cadherin distribution (Price et al., 2002). Similarly, in the cerebellum progenitors have different distributions of cadherins, and neurons appear to migrate through the circuits which express the corresponding cadherin (Redies et al., 2011). The subregions of cerebellar cortex that express certain cadherin are connected to the brain regions which express the same cadherin. In the developing hindbrain, r6-7 cells expressing *Cdh6* appear to segregate from the cells in r4-5 which are not expressing *Cdh6* (Inoue et al., 1997, Inoue et al., 2009). In the chicken optic tectum, three tectofugal pathways, the tectothalamic, tectobulbar, and tectoisthmic tracts, innervate from the superior colliculi. They are divided into subtracts based on differential expression of *N-Cdh*, *Cdh7*, *Cdh6b* and *R-Cdh* (Treubert-Zimmermann et al., 2002). Axons that ectopically express a certain cadherin, selectively navigate into subtracts that express a matching type of cadherin. Also, in the cortico-striatal boundary of telencephalon, *Cdh6* is expressed the striatum and R-Cdh in the cerebral cortex (Inoue et al., 2001). The

neurons are segregated according to the cadherin they express (Halbleib and Nelson, 2006). Also *Cdh7* and *Cdh20* are expressed in the certain brain regions during the early development of the rat CNS (Takahashi and Osumi, 2008). The activity of classical cadherins can be regulated either transcriptionally or post-translationally by regulating protein transportation to the cell surface and its turnover state. The level of expression in the cell surface may also function as a selector tool between different cellular subtypes (Gumbiner, 2005, Halbleib and Nelson, 2006).

Neurite outgrowth and synaptogenesis. Dynamic activity of cadherin expression is required for neurite outgrowth and pathfinding, synaptogenesis and also for modifying synaptic plasticity (Takeichi, 2007, Suzuki and Takeichi, 2008). N-Cdh and R-Cdh are able to promote neurite outgrowth through *cis* activation of Fgfr and the PLCγ-pathway (Sanchez-Heras et al., 2006). Moreover, Cdh11 appears to stimulate axon elongation and Cdh13 functions as a repulsive molecule guiding growth cone navigation. Abolishing *N-Cdh* expression in chick or *Drosophila* caused mistargeting of axons, whereas hippocampal projections of the *Cdh11^{null}* mice appeared normal (Manabe et al., 2000, Halbleib and Nelson, 2006). Especially, the type II cadherins appear to be expressed in neuronal groups which are connected to each other (Takeichi, 2007). Many cadherins are expressed in forming synapses, such as R-Cdh and Cdh7, and after synaptogenesis cadherins are involved in maintaining or remodelling synaptic contacts (Halbleib and Nelson, 2006).

2.3.2.2. Cadherin-22, midbrain-hindbrain boundary specific cadherin

The midbrain-r1 specific *Fgfr1* mutants (*Fgfr1^{cko}*) have cell adhesion defects in the MHB (see above) (Trokovic et al., 2003). *Cdh22* expression is downregulated in *Fgfr1^{cko}* mutants, which suggest, that Fgf signalling is able to modify *Cdh22* expression. Cdh22 belongs to the classical cadherins, but it lacks the HAV tripeptide from its first EC domain (Sugimoto et al., 1996). This motif is replaced by a QAR (Glutamine-Alanine-Arginine) tripeptide and, thus, it belongs to the type II classical cadherins (Kitajima et al., 1999). Moreover, homology of Cdh22 to the type II cadherins, such as Cdh11, Cdh6 and Cdh8, is higher than to the type I cadherins, such as E-Cdh or N-Cdh. Alternative splicing produces two different Cdh22 isoforms: the long-type and short-type Cdh22. All five EC domains and transmembrane domains are similar in these isoforms, whereas intracellular domains differ (Sugimoto et al., 1996). The cytosolic part of the long-type Cdh22 includes the catenin-binding domain, whereas this domain is lacking from the short-type. The short-type of Cdh22 has been shown to promote cell survival in gonocytes of new born rats, and this action is mediated through JAK-STAT signalling (Wu et al., 2003, Wu et al., 2005). Thus, the long-type and the short-type Cdh22 may be involved in separate processes during embryonic development. *Cdh22* is expressed in the brain, spinal cord and limb buds at E10.5 (Kitajima et al., 1999). In the brain, the strongest expression has been identified in the forebrain and the MHB. In the midbrain-hindbrain boundary, *Cdh22* is expressed on both sides of the border (Kala et al., 2008).

2.3.3. Cell adhesion molecules cooperate with Fgfrs in the developing CNS

Cooperation between CAMs and Fgfrs appears to promote several processes during neuronal development. N-Cam, N-Cdh and L1 are able to stimulate axonal growth (Williams et al., 1994). This stimulation is mediated through Fgfr and is conserved evolutionarily (Williams et al., 2001). In some cancers, CAMs stabilize Fgfr on the cell surface, or cause sustained activation of Fgfr and downstream pathways (Cavallaro et al., 2001, Suyama et al., 2002). Fgfrs have sequence homology with N-cadherin and N-Cam in the II Ig domain, and it is connected to the acid box (Doherty and Walsh, 1996). If the *cis* interaction through this homology domain or acid box is prevented, CAMs fail to stimulate outgrowth of neurites. Fgfr I and II Ig domains are required for cis interaction with N-Cdh EC4 and EC5 domains, whereas the III Ig domain, which is necessary for ligand binding, is not sufficient for CAM-Fgfr adhesion (Williams et al., 2001). The Fgfr1 C isoform is able to interact with CAMs, and the acid box appears to be the most crucial mediator in CAM-Fgfr interaction (Sanchez-Heras et al., 2006). Interestingly, deletion of this motif is enabled by alternative splicing and, thus, indicates possibility for ligand selection in different systems. Moreover, Fgfr1 is directly interacting with N-Cdh and N-Cam, and different isoforms of N-Cam do not affect the binding affinity (Sanchez-Heras et al., 2006). In addition, Cdh11 induces neurite outgrowth through Fgfr activity (Boscher and Mege, 2008). The activity is mediated by PLCγ and PI3 kinase pathways. Fgf signalling appears to also modulate Ephrin signalling (Lee et al., 2009).

3. AIMS OF THE STUDY

The aims of this study were to elucidate redundant functions of Fgfrs in the developing midbrain and anterior hindbrain, and analyse where and when the Fgf-regulated adhesion molecule, *Cadherin22*, is expressed and how it functions during the development of the central nervous system.

The specific aims were:

1.   To study the function of *Fgfr2* and *Fgfr3* in the midbrain and anterior hindbrain

2.   To study cooperation and potential redundancy of *Fgfr1*, *Fgfr2*, and *Fgfr3* in the developing midbrain and anterior hindbrain

3.   To clarify the role of Fgf signalling during the development of the midbrain dopaminergic neurons

4.   To analyse the role of Fgf signalling in the regulation of cell cycle exit and postmitotic neuronal differentiation in the midbrain

5.   To study the function of *Cadherin22*, a potential FGF signalling target, in the regulation of cell-adhesive properties at the midbrain-hindbrain border

4. MATERIALS AND METHODS

The materials used in this study are listed in Table 1 and 2.

Table 1. Transgenic mouse lines

Transgenic allele		References	Publication
Fgfr1^{flox}	Conditional allele	(Trokovic et al., 2003)	II,III
Fgfr^{IIICn}	Null allele	(Partanen et al., 1998)	III
Fgfr2^{floxa}	Conditional allele	I	I
Fgfr2^{floxb}	Conditional allele	(Yu et al., 2003)	II, III
Fgfr3^{null}	Null allele	(Colvin et al., 1996)	I-III
R26R	R26R reporter allele	(Soriano, 1999)	III
En1-Cre	Cre in En1 locus	(Kimmel et al., 2000)	I-III
Shh-Cre	Cre in Shh locus	(Harfe et al., 2004)	III
Cdh22^{null}	Null allele	(Turakainen et al., 2009)	IV

Table 2. PCR primers for genotyping

Transgen- ic allele	Primers		Size of product
Fgfr1^{flox}	R1-1	5' AATAGGTCCCTCGACGGTATC 3'	flox 210 bp
	R1-2	5' ATGCAAGTTGGCTCTGGAGT 3'	wt ~300bp
	R1-3	5' GGAAGTCGCTCTTCTTGGTG 3'	
Fgfr2^{flox}	R2-1	5' ATAGGAGCAACAGGCGG 3'	flox: 207 bp
	R2-2	5' TGCAAGAGGCGACCAGTCAG 3'	wt: 142 bp
Fgfr3^{null}	R3-1	5' GGGCTCCTTATTGGACTCGC 3'	null: 221bp
	R3-2	5'AGGTATAGTTGCCACCATCGGAGGG 3'	wt: 322bp
	R3-3	5' TGCTAAAGCGCATGCTCCAGACTG 3'	
En1-Cre	Cre5'	5' AATCTCCCACCGTCAGTACG 3'	cre 500bp
Shh-Cre	Cre3'-	5' CGTTTTCTGAGCATACCTGGA 3'	wt -
R26R	5' OIMR315 5' GCCAAGAGTTTGTCCTCAACC 3'		R26R 320 bp
	3' OIMR316 5' GGAGCGGGAGAAATGGATATG 3'		wt 650 bp
	3' OIMR883 5' AAAGTCGCTCTGAGTTGTTAT 3'		
Cdh22^{null}	PBCT5'	5' GGATGCCCTCTCACACCCTCC 3'	wt 337 bp
	TP3'	3' GGGAACACAGAGAGACCCAGAAGC 3'	null 627bp*
	TD3'a	3' CCGTGTCCCTTCTCTAGTGCCAC 3'	

*PCRs for wt and null allele were done separately

The methods used in this study are listed in Table 3. The probes used in situ hybridizations are listed in Table 4 and antibodies used for immunohistochemistry in Table 5. From Publication I Tables 4 and 5 includes only probes and antibodies, which were used in Helsinki.

Table 3. Methods used in this study

Method	Reference	Publication
Radioactive section in situ hybridization	(Wilkinson and Green, 1990)	I-IV
Non-radioactive section in situ hybrization	(Jukkola et al., 2006)	II,IV
Whole mount in situ hybridization	(Henrique et al., 1995)	I-IV
Immunohistochemistry	(Jukkola et al., 2006), III	I-IV
PCR genotyping	(Trokovic et al., 2003), See table 2	I-IV
TUNEL assay for sections	(Trokovic et al., 2005)	II
TUNEL assay for whole embryos	(Chi et al., 2003)	II
BrdU incorporation	(Trokovic et al., 2005)	II, III
Cumulative BrdU incorporation analysis	(Takahashi et al., 1995), (Calegari et al., 2005)	III
BrdU-EdU incorporation	III	III
Pair-cell assay	(Shen et al., 2002)	III
Semi-Quantitative PCR	IV	IV
Statistical similarity analysis	(Rita and Ekholm, 2007)	III
Neurofilament staining	(Trokovic et al., 2003)	II,IV
Semi-thin sections	II	II
Electronmicroscopy	III	III
Generation of chimeric embryos	(Nagy et al., 2002,) III	III

CONSTRUCTION OF A TARGETING VECTOR AND INDUCTION OF MUTAGENESIS IN CDH22 LOCUS (IV)

Targeting vectors for the *Cdh22* locus were produced by two different methods: transposon technique (Turakainen et al., 2009) and Ret/ET technique (Zhang et al., 2000, Muyrers et al., 2001). We used both targeting vectors successfully for targeting ES-cells. The ES cells were aggregated with morula stage embryos. The ES cells targeted with both methods were able to produce chimeric mice. Transposon-based mutation in the *Cdh22* locus was transferred to the germ. Therefore, the allele generated by transposon technique was used in the characterization of the *Cdh22^null^* phenotype.

PROBES

Table 4. Following probes were used for situ hybridizations in this study

Probe	Reference/Source	Publication
En1	(Trokovic et al., 2003)	I,II
Otx2	(Acampora et al., 1995)	I,II,IV
Fgf8	(Crossley and Martin, 1995)	I,II,IV
Sprouty1	(Trokovic et al., 2003)	I-III
Erm	(Trokovic et al., 2005)	I-III
Gbx2	(Trokovic et al., 2003)	I,II
Pea3	a gift from Sylvia Arber	II,
Fgf17	(Jukkola et al., 2006)	II
Pax6	a gift from Peter Gruss	II
HoxA2	(Trokovic et al., 2005)	II
Dat	a gift from Wolfgang Wurst	II
Fgfr1Δ	(Trokovic et al., 2003)	II-III
Aldh1	a gift from Francois Guillemot	II
Pitx3	(Jukkola et al., 2006)	II
Ngn2	(Jukkola et al., 2006)	II-III
Mash1	(Jukkola et al., 2006)	II-III
Gata3	(Lillevali et al., 2004)	II
Pou4f1	a gift from Siew-Lang Ang	II, IV
Lmx1a	IMAGE 317647	II
Nurr1	a gift from Thomas Perlmann	II
Wnt1	(Trokovic et al., 2005)	II
Drapc1	(Jukkola et al., 2004)	II
Shh	a gift from Irma Thesleff	II
Gli1	a gift from Irma Thesleff	II
Axin2	a gift from Irma Thesleff	II
CyclinD1	(Trokovic et al., 2005)	II-III
CyclinD2	(Trokovic et al., 2005)	II-III
p21	(Trokovic et al., 2005)	II
Sox3	(Jukkola et al., 2006)	II,IV
Hes5	a gift from Irma Thesleff	II

Fgfr2Δ	(Trokovic et al., 2005)	II
DBH	see publication I	II
Sert	see publication I	II
Islet	a gift from Wolfgang Wurst	II, IV
Pet1	(Jukkola et al., 2006)	II
PhoxA2	(Jukkola et al., 2006)	II
Hes1	A gift from Irma Thesleff	III
Dll1	IMAGE p968GO3104D	III
Dusp6	(Jukkola et al., 2006)	III
Tis21	A gift from Wieland Huttner	III
Jagged1	IMAGE IRAVp968611131D6	III
CyclinB1	IMAGE 3971364	III
Cdh22	IMAGE UI-M-BH4-azf-e10-o-ui	IV
Cdh11	IMAGE clone 4035346	IV
Cdh6	IMAGE IRCLp5011G0820D	IV
Cdh8	IMAGE IRAVp968E01116D	IV
Gad1	RZPD IRAV p968 M67D6	IV
Vglut2	(Guimera et al., 2006)	IV
Lmx1b	a gift from Horst Simon	IV

ANTIBODIES

Table 5. Following primary antibodies were used for immunohistochemistry in this study

Antigen	Dilution	Source	Publi-cation
mouse anti-Th	1:500	Millipore	II
rabbit anti-Lmx1A	1:300	gift from Michael German,	II,III
mouse anti-HuC/D	1:500	Invitrogen	II, III
rabbit anti-Pitx3	1:400	Invitrogen	II
rabbit anti-Aldh1	1:500	Abcam	II
rabbit- anti Sox2	1:500	Millipore	II,III
rabbit anti-Sox3	1:500	gift from Thomas Edlund	II
mouse anti-BrdU	1:400	GE Healthcare	II, III
rabbit anti-p57	1:500	Neomarkers	III
rabbit anti-β-galactosidace	1:500	MP Biomedicals	III
rabbit anti-Par3	1:500	Millipore	III
mouse anti-aPKCλ	1:500	BD Biosciences	III
mouse anti- β-catenin	1:500	BD Biosciences	III
mouse anti-Zo1	1:500	Zymed	III
rabbit anti-Zo2	1:500	Santa Cruz Biotechnology	III
goat anti-Fgf8	1:400	R&D systems	III
mause anti-Pancadherin	1:500	Abcam	III
rabbit anti-Laminin	1:800	Abcam	III
mouse anti-Nestin	1:500	Millipore	III
rabbit anti-γtubulin	1:500	Sigma	III
rabbit anti-phospho-Erk1/2	1:100	Cell Signalling Technologies	III
rabbit anti-CyclinD1	1:400	Neomarkers	III
mouse anti-Nucleonin	1:50	Santa Cruz Biotechnology	III
rabbit anti-Phospho-histone3 (Ph3)	1:500	Upstat	III
rabbit anti-serotonin (5-HT)	1:5000	Immunostar	IV
rabbit anti-GFAP	1:500	Millipore	IV
mouse anti-Islet1	1:200	Developmental Studies Hybridoma Bank	IV
mouse anti-Gad67	1:500	Millipore	IV
rabbit anti-Vglut2	1:1000	Sigma	IV

5. RESULTS AND DISCUSSION

5.1. Fgf receptors redundantly regulate patterning of the midbrain and hindbrain (I-II)

5.1.1. Fgfr2 and Fgfr3 are not essential for proper patterning of the midbrain and anterior hindbrain (I)

Phenotypic differences between the *Fgfr1^{cko}* and the *Fgf8^{cko}* mutants (see Review of the Literature) suggests that besides Fgfr1, other Fgf receptors such as Fgfr2 and Fgfr3 may mediate Fgf signals in the midbrain and anterior hindbrain region. *Fgfr2* and *Fgfr3* are not expressed or expression is very weak in the MHB at E8.5-E11.5 and their expression form a concentration gradient being strongest in the anterior midbrain and posterior r1 (Blak et al., 2005, Trokovic et al., 2005). As *Fgfr2* and *Fgfr3* are also expressed in the midbrain-anterior hindbrain territory, we wanted to elucidate the function of these two receptors during midbrain and anterior hindbrain development. To prevent gastrulation defects, which appear in the *Fgfr2^{null}* mutants, we used a conditional mutagenesis approach to study the function of Fgfr2. The *Fgfr3^{null}* mice are viable and could be used for studying brain development (Colvin et al., 1996). The *Fgfr2^{cko}* and the *Fgf3^{null}* mice lacked the major anatomical brain defects at E18.5. We checked the expression of genes that are important for the development of the MHB, *En1*, *Pax2*, *Otx2*, *Gbx2*, *Fgf8*, *Shh* and *Wnt1*, in the *Fgfr2^{cko}* and *Fgfr3^{null}* mutants at E12.5. The MHB specific genes as well as Fgf signalling target genes, such as *Sprouty* and *Erm*, were expressed in normal patterns in these mutants (see Fig. 2, 4, 5 in I). In addition, although one allele of *Fgfr1* was deleted together with *Fgfr2*, no obvious defects could be observed in the expression of these genes at E9.5 (see Suppl. Fig. 1 in I). Dorso-ventral patterning of the *Fgfr2^{cko}* midbrain was studied by specific markers of the midbrain domains, such as *Th* (m7), *Nkx6.1* (m6), *Nkx2.2* (m4-m5, m2) and *Pou4f1* (m6, m2-m1), at E12.5. These cell populations were not changed in the *Fgfr2^{cko}* mutants indicating normal dorso-ventral patterning in the *Fgfr2^{cko}* mutants (see Fig. 2 in I). No defects could be detected in the development of either dopaminergic, serotonergic, gabaergic or cranial motor nuclei neurons in the *Fgfr2^{cko}* or *Fgfr3^{null}* mutants (see Fig.3 and 4 in I). In the adults, oligodendrocytes also appeared in normal numbers (see Fig.3 and 4 in I).

These findings revealed that the development of the midbrain and anterior hindbrain occurs normally without *Fgfr2* or *Fgfr3*. Since at least Fgfr2 is required for neurite outgrowth (Sato et al., 2001b), some minor changes that could not be observed in this study might still occur in these mutants later in brain development or in the adulthood. In the developing kidney, deletion of either *Fgfr1* or *Fgfr2* alone did not affect the early development of the ureteric bud (Poladia et al., 2006, Bates, 2007). However, the deletion of both *Fgfr1* and *Fgfr2* from metanephric mesenchyme caused failures in the ureteric bud elongation and branching morphogenesis. Similarly, Fgfr2 and Fgfr3 might have redundant functions together with *Fgfr1* during the brain development.

5.1.2. Fgf receptors cooperate to regulate the development of the midbrain and rhombomere1 (II,III)

To elucidate the cooperative role of the Fgfrs, we combined different variations of *Fgfr* mutant alleles. The inactivation of conditional alleles (*Fgfr1^{cko}* and *Fgfr2^{cko}*) was localized to the midbrain-rhombomere 1 region by expressing Cre-recombinase from the *Engrailed1* locus. We created the following combinations: *Fgfr2^{cko};Fgfr3^{null}* mutants, *Fgfr1^{cko};Fgfr2^{cko}* mutants, *Fgfr1^{cko};Fgfr3^{null}* mutants and *Fgfr1^{cko};Fgfr2^{cko};Fgfr3^{null}* mutants.

5.1.2.1. Loss of several Fgfrs leads to altered brain morphology (II)

We analysed the anatomical structures of Fgfr double and triple mutant brains. We could not detect any defects in the *Fgfr2^{cko}; Fgfr3^{null}* mutant brains, similar to *Fgfr2^{cko}* and *Fgfr3^{null}* single mutants (Fig. 7B-D). This finding indicates a prominent role of *Fgfr1* in the development of the midbrain and anterior hindbrain. However, the *Fgfr1^{cko};Fgfr2^{cko}* brains showed relatively large alterations in the midbrain-r1 territory (Fig. 7G). The dorsal structures, such as the SC and the IC as well as the cerebellum, were lost in the *Fgfr1^{cko};Fgfr2^{cko}* mutants. Only the most anterior structure of the dorsal midbrain, posterior pretectum, remained in the *Fgfr1^{cko};Fgfr2^{cko}* mutants. Although, some of the ventral midbrain-r1 tissue still was present at E18.5, also ventral regions were altered in these mutants. In contrast, the *Fgfr1^{cko};Fgfr3^{null}* mutant brains largely resembled the *Fgfr1^{cko}* mutant (Fig. 7E) brains and lacked the IC and the vermis of the cerebellum (Fig. 7F; Trokovic et al., 2003). Nevertheless, removal all of these three receptors from the midbrain and anterior hindbrain resulted in the most severe phenotype including loss of the posterior pretectum of the dorsal midbrain (Fig. 7H). These *Fgfr1^{cko};Fgfr2^{cko};Fgfr3^{null}* mutant brains most closely resemble the *Fgf8^{cko}* mutant phenotype. Interestingly, the target genes of Fgf signalling, such as *Erm*, *Pea3*, *Sprouty1*, *Fgf8* itself and *En1,* showed a gradual downregulation, which corresponded to reduced transduction of Fgf signalling (see Fig. 1 in II). Whereas the *Fgfr1^{cko}* mutants showed target gene downregulation mainly in the specific boundary cell population, the *Fgfr1^{cko};Fgfr2^{cko}* totally lacked the dorsal expression domains and the target genes were also downregulated ventrally (see Fig. 1 in II, Trokovic et al., 2005). The ventral defects were even more obvious in the midbrain-r1 territory of *Fgfr1^{cko};Fgfr2^{cko};Fgfr3^{null}* mutants (see Fig. 1 in II). Hence, all three Fgf receptors, Fgfr1, Fgfr2 and Fgfr3, are needed for signal transduction in the midbrain and anterior hindbrain regions.

These findings indicate that Fgfr1 is functionally the most important Fgf receptor in the midbrain-r1 region. Fgfr1 is an essential mediator of Fgf signalling in the specific midbrain-hindbrain boundary cell population. Moreover, Fgfr1 also has pivotal roles in other parts of the midbrain and r1, because inactivation of both *Fgfr2* and *Fgfr3* do not lead alterations in the midbrain-r1 development. However, Fgfr2 and Fgfr3 are also needed for mediating Fgf signalling within the midbrain and r1. Large differences between the *Fgfr1^{cko}* and the *Fgfr1^{cko};Fgfr2^{cko}* mutant phenotypes revealed that Fgfr2 transduces Fgf signals in the midbrain-r1 region excluding the specific midbrain-hindbrain boundary cell population, in which *Fgfr1* is crucial. *Fgfr3* has only a minor role, which is consistent with the limited *Fgfr3* expression domain. *Fgfr3* expression always overlaps with *Fgfr2* expression and, thus, *Fgfr3* is not crucial for the development of the midbrain-r1 territory if the other *Fgfr* are normally expressed. In the absence of *Fgfr1* and *Fgfr2*, *Fgfr3* is sufficient to mediate Fgf8 signal in the
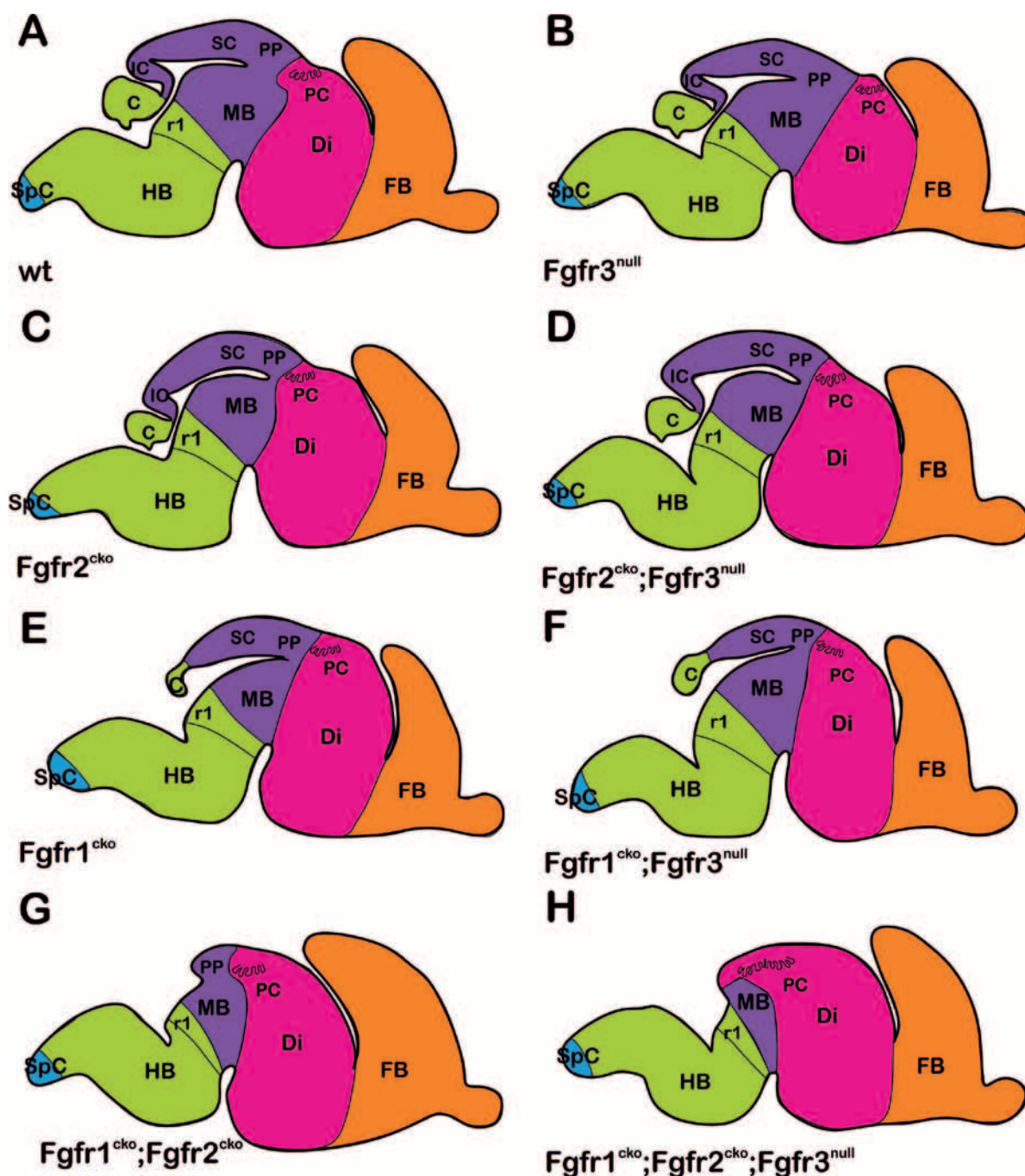
Figure 7. **Morphological differences between distinct Fgfr genotypes.** Normal structure of the E18.5 brain **(A)**. No clear defects in the *Fgfr3^null* **(B)**, *Fgfr2^cko* **(C)** and *Fgfr2^cko;Fgfr3^null* **(D)** brains. *Fgfr1^cko* **(E)** and *Fgfr1^cko;Fgfr3^null* **(F)** lack the vermis of the cerebellum and the inferior colliculi from the dorsal midbrain. *Fgfr1^cko;Fgfr2^cko* lack the cerebellum, and the dorsal midbrain structures such as the inferior colliculi, the superior colliculi ,as well as some ventral structures **(G)**. Diencephalic tissue is expanded in the dorsal area. *Fgfr1^cko;Fgfr2^cko;Fgfr3^null* phenotype is most severe **(H)**: All the midbrain and rhombomere1 derived dorsal structures are lost. The diencephalic posterior commissure is clearly enlarged and diencephalic tissue replaces the dorsal midbrain tissue. Also ventral structures are severely affected. All schematic views of distinct genotypes are drawn based on the histological section and, thus, brains show slight differences. The borders of the brain regions are drawn based on Allen Brain Atlas. FB forebrain, Di diencephalon, MB midbrain, HB hindbrain, SpC spinal cors, PP posterior pretalamus, SC superior colliculi, IC inferior colliculi, c cerebellum, r1 rhombomere 1.

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ventral r1 and the most anterior midbrain, where it is expressed. Thus, some anterior structures, such as the posterior pretectum are still present in the *Fgfr1^{cko};Fgfr2^{cko}* mutants but are lost from the *Fgfr1^{cko};Fgfr2^{cko};Fgfr3^{null}* mutants. Similarly, in the forebrain cooperation of Fgfr is needed for early specification of the neural progenitors and, there Fgfr1 also has a dominant role in the regulation of early patterning and differentiation (Gutin et al., 2006). All Fgfr c isoforms seem to bind Fgf8 family members with similar affinity (Olsen et al., 2006). Thus, the differences between *Fgfr* mutant phenotypes are likely caused by divergent expression domains rather than variation in Fgfr binding affinity.

5.1.2.2. Fgf receptors regulate antero-posterior patterning in the midbrain and anterior hindbrain region (II)

The midbrain and r1 region can be restricted based on *Pax6* and *HoxA2* expressions, which are expressed in the diencephalon and r2, respectively. Based on in situ hybridizations with *Pax6* and *HoxA2*, *Fgfr1^{cko};Fgfr2^{cko}* mutants still have some midbrain-r1 tissue left (see Fig. 3 in II). The midbrain (*Otx2* expressing region) border was also shifted caudally and *Gbx2* expression was downregulated in the anterior r1. Fgf8 has also been shown to regulate *HoxA2* expression (Irving and Mason, 2000) and, thus, the caudal border of r1 seem to be slightly affected in the *Fgfr1^{cko};Fgfr2^{cko}* mutants . Expression of another signalling molecule, *Wnt1*, was lost from the MHB, but ventral floor plate-specific expression and dorsal roof plate expression patterns still remained (see Fig. 7 in II). Similarly, ventral *Shh* expression was not abolished in the embryonic *Fgfr1^{cko};Fgfr2^{cko}* brain (see Fig. 7 in II). Other neuronal markers, such as *Lmx1a* (m7), *Pou4f1* (m6), *Gata3* (m5-m3), were expressed in approximately correct positions indicating normal dorso-ventral patterning of the *Fgfr1^{cko};Fgfr2^{cko}* mutant midbrain (see Fig. 6 in II).

5.1.2.3. Fgf receptors promote cell survival in the dorsal midbrain (II)

The loss of the midbrain and cerebellum structures in the *Fgf8^{cko}* is primarily the result of extensive cell death between E8.5-E10.0 (Chi et al., 2003). The midbrain region was reported to undergo apoptosis before the r1 region. Similarly, we observed ectopic apoptosis in *Fgfr1^{cko};Fgfr2^{cko}* and *Fgfr1^{cko};Fgfr2^{cko};Fgfr3^{null}* mutants from E8.5 onwards (see Fig. 2 in II). We did not measure temporal differences between the midbrain and the r1, but cell death seemed to be more prominent in the midbrain region in early embryos (E8.5-E9.0), whereas more apoptotic cells were identified in more caudal locations of the *Fgfr1^{cko};Fgfr2^{cko}* mutants at E9.5. As in the *Fgf8^{cko}* (Chi et al., 2003), ectopic cell death is also concentrated in the dorsal regions in the *Fgfr1^{cko};Fgfr2^{cko}* mutants (see Fig. 2 in II). Fgf signalling has also been shown to promote the cell survival in developing branchial arches and olfactory epithelium (Trumpp et al., 1999, Kawauchi et al., 2005). Interestingly, either the loss or enhancement of Fgf8 signalling caused increased apoptosis in the forebrain, whereas a reduction of Fgf8 signalling promoted cell survival (Storm et al., 2003). Thus, Fgf signalling has dosage and maybe context dependent functions in cell survival. Loss of the dorsal midbrain-r1 structures in *Fgfr1^{cko};Fgfr2^{cko}* and *Fgfr1^{cko},Fgfr2^{cko};Fgfr3^{null}* mutants is likely a primary outcome of programmed cell death. Thus, the appropriate amount of Fgf signalling is absolutely required for development of dorsal structures. However, in the ventral regions apoptosis could not explain all of the alterations observed.

5.1.3. The development of the midbrain and the anterior hindbrain neuronal populations is altered in the Fgfr mutants (II, III)

In the *Fgf8^{cko}* mutants, some neuronal populations, such as dopaminergic SN and VTA, noradrenergic LC and cranial motoneurons III and IV, were lost. Thus, we studied the existence of these populations also in the *Fgfr1^{cko};Fgfr2^{cko}* and the *Fgfr1^{cko},Fgfr2^{cko};Fgfr3^{null}* mutants. At E18.5, the TH expressing VTA, SN and LC were lost (see Fig. 4 in II). Serotonergic neurons from the raphe nuclei were also lacking, as well as the oculomotor and the trochlear motor neurons (see Suppl. Fig. 2 in II). Although the mDA neurons were lost at E18.5, the early markers of the mDA progenitors, such as *Aldh1* and *Pitx3*, were still expressed, but their expression was weaker and spread out at E10.5-E11.5 (Fig. 5 in II). Interestingly, *Nurr1* expression was even elevated in the mutants (see Fig. 6 in II). The proneural genes, *Ngn2* and *Mash1*, were also still expressed in the ventral midbrain suggesting on-going neuronal differentiation (see Fig. 6 in II). Indeed, some TH-expressing cells could be detected at E12.5 and even E15.5 (see Fig. 4 in II). However, these cells failed to express Pitx3 or *Dat*, genes typical for functional mDA neurons. These results suggest that whereas early differentiation of the mDA precursors is normal in the *Fgfr1^{cko};Fgfr2^{cko}* mutants, the original number of the mDA precursors is decreased and final maturation and maintenance of the mDA fate fails.

Recent results from our laboratory suggest that the decreased number of mDA precursors in *Fgfr1^{cko};Fgfr2^{cko}* mutants is caused by alterations in antero-posterior patterning of the mDA domain (Lahti et al., 2012). Normally, dopaminergic neuron progenitors in the midbrain floor plate receive Fgf from the IsO. The Fgf signal guides the patterning of these progenitors to become the midbrain dopaminergic neurons. When Fgf signalling is lost from the midbrain region, these neuronal progenitors adopt features of diencephalic dopaminergic progenitors. These diencephalic progenitors develop independent of Fgf signalling. They arise from an Lmx1a positive progenitor population, differentiate and start TH expression earlier (E10.5) than midbrain DA precursors (E11.5; Lahti et al., 2012). They are expressed in a mixed population together with *Pou4f1*-positive cells. In the *Fgfr1^{cko};Fgfr2^{cko}* mutants, ventral midbrain DA progenitors and precursors resemble and express many genes similarly to these diencephalic DA neurons. Interestingly, TH expression of the diencephalic DA neurons may be lost in a manner similar to the *Fgfr1^{cko};Fgfr2^{cko}* mutant mDA cells (see Fig. 2 in II; Lahti et al., 2012). This study also elucidated the later role of Fgf signalling in maintenance of the mDA neuron fate. When *Fgfr1* and *Fgfr2* were deleted by using specific Cre lines later in *Dat* (expressed E12.5 onwards) or *TH* expressing cells, no obvious alterations in the mDA neurons was observed (Lahti et al., 2012). The adult mice were viable and lacked major behavioural abnormalities. However, the long term survival of Fgfr deficient DA neurons was not analysed in this study. Thus, loss of mDA neurons in the *Fgfr1^{cko};Fgfr2^{cko}* mutants is caused by abnormal patterning rather than a failure in differentiation or later maintenance of dopaminergic fate.

5.2. Loss of Fgf signalling causes premature differentiation
of neural progenitors in the ventral midbrain (II-III)

5.2.1. The number of proliferative neural progenitors is reduced in Fgfr mutants (II-III)

Defects in neuronal populations in *Fgfr1^{cko};Fgfr2^{cko}* mutants might be due to abnormal neural progenitor proliferation or neurogenic cell cycle exit. We addressed these questions by analysing the factors involved in cell cycle progression, maintenance of a proliferative state and initiation of neurogenesis. Indeed, *CyclinD1* and *CyclinD2* appeared to be downregulated in the *Fgfr1^{cko};Fgfr2^{cko}* and the *Fgfr1^{cko};Fgfr2^{cko};Fgfr3^{null}* mutants already at E9.5 (see Fig. 8 in II). The dorsal region was more affected than the ventral domain, although *CyclinD1* and *CyclinB1* mRNA levels were downregulated in the ventral midbrain at E11.5 (see Suppl.Fig. 4 in III). In addition, the proliferative layer of the ventral midbrain was thinner (Fig 8A) especially in the ventro-lateral regions after the beginning of neurogenesis (E10.5-E11.5). BrdU-positive nuclei in the *Fgfr1^{cko};Fgfr2^{cko}* mutants were located closer to the ventricular surface in ventro-lateral regions (see Fig 9 in II). The most ventral domain, from which the mDA neurons are derived, was less affected indicating that some other signalling pathways besides of Fgfs are involved in the regulation of floor plate cell proliferation.

To follow the fate of proliferating neural progenitors, we performed a BrdU-pulse-chase study. The BrdU pulses were given at E9.5 and E10.5 and embryos were dissected 24 hours later. These experiments verified premature neurogenesis in the Fgfr1^{cko};*Fgfr2^{cko}* and *Fgfr1^{cko};Fgfr2^{cko};Fgfr3^{null}* mutants (see Fig 3. in III). Premature neurogenesis was apparent in both Lmx1a-positive and Lmx1a-negative regions in the ventral midbrain and consistent with previous results it was more pronounced in the ventro-lateral domain. Premature differentiation was observed in the *Fgfr1^{cko};Fgfr2^{cko};Fgfr3^{null}* mutants already at E9.5 and by E11.5 the difference between controls and mutants was pronounced (see Fig. 3 in III). Increased neurogenesis has been suggested to result from lengthening of the cell cycle and especially the G1 phase (Lange and Calegari, 2010, Salomoni and Calegari, 2010). *Cdk4/cyclinD1* overexpression in neural stem cells shortens the G1, delays neurogenesis, and promotes the generation and expansion of basal progenitors, and inhibition of *Cdk4/CyclinD1* expression lengthens the G1 and increases neurogenesis (Lange et al., 2009). Thus, we calculated cell cycle length based on cumulative BrdU labelling. The *Fgfr1^{cko};Fgfr2^{cko}* mutants showed a small increase in cell cycle length, but based on statistical similarity analysis the increase was not enough to explain the loss of proliferative progenitors in the *Fgfr1^{cko};Fgfr2^{cko}* mutants (see Fig 3. and Suppl. Fig.3 in III). Thus, cell cycle progression was not markedly affected in the *Fgfr1^{cko};Fgfr2^{cko}* mutants although mRNA levels of *CyclinD1* and *CyclinB1* were decreased (see Suppl.Fig. 4 in III). Interestingly, in contrast to the mRNA levels, CyclinD1 protein level remained relatively normal in the *Fgfr1^{cko};Fgfr2^{cko}* mutants (see Suppl. Fig. 4 in III). This finding indicates that the lowered mRNA level was still enough to a produce sufficient amount of cell cycle regulator proteins to maintain cell cycle progression. Interestingly, the S phase in the wild-type controls was longer than the *Fgfr1^{cko};Fgfr2^{cko}* mutants, which have increased neurogenesis. The progenitors that undergo symmetric, proliferative divisions require the longer S-phase than the progenitors that divide by neurogenic divisions, to ensure proper synthesis of DNA (Arai et al., 2011).

Next, we wanted to clarify the reason for premature neurogenesis in the Fgfr mutants. The decision to proliferate or differentiate is regulated by a balance between proliferative factors,
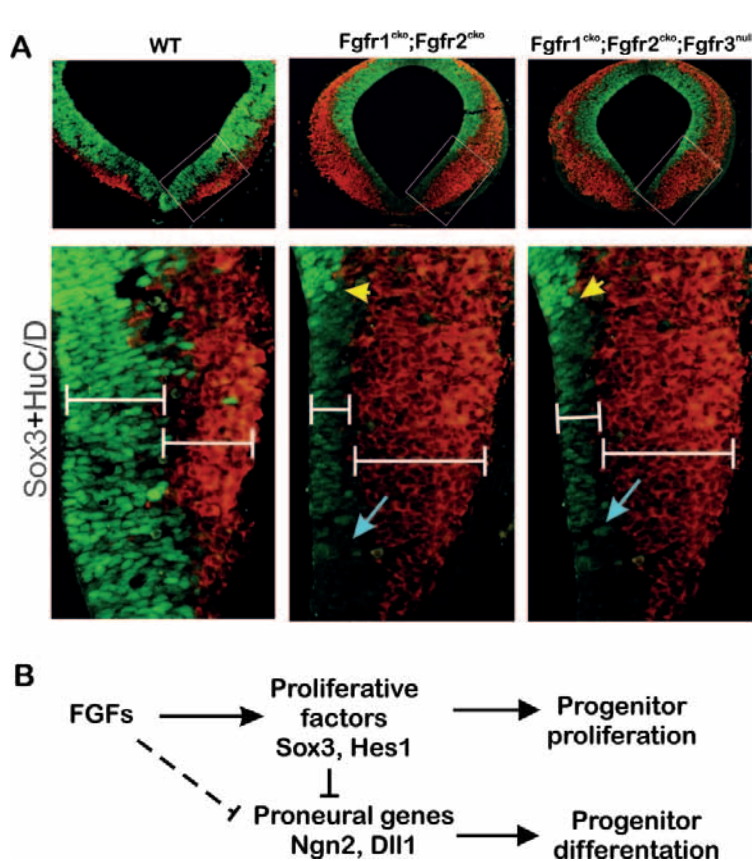
Figure 8. **Fgf signalling promotes proliferation of the neural progenitors.** The *Fgfr1^{cko};Fgfr2^{cko}* and the *Fgfr1^{cko};Fgfr2^{cko};Fgfr3^{null}* mutants have a thinner ventro-lateral ventricular zone and thicker mantle zone combined to wild type controls and, thus, decreased number of the neural progenitors **(A)**. Proliferative factors, such as Sox3, were downregulated in mutants **(A)**. Our results suggest a model, in which Fgfs promote expression of proliferative factors, such as Sox3 and Hes1, and, thus, proliferation **(B)**. On the other hand, Fgfs inhibit, directly or indirectly through lateral inhibition, the expression of proneural genes and neural differentiation. Blue arrow in **A** indicates the decreased Sox3 expression and yellow arrowhead the dorsal border where Sox3 expression changes normal.

such as *SoxB1* and *Hes* genes, and proneural genes. The *SoxB1* group contains *Sox1-3*, which promote the undifferentiated state of the neural progenitors (Wegner and Stolt, 2005, Wegner, 2011). In the ventral midbrain, the expression of *Sox2* and *Sox3* normally overlaps. Interestingly, in the *Fgfr1^{cko};Fgfr2^{cko}* mutants Sox3, but not Sox2, was downregulated in the ventral midbrain (Fig. 8A). The Hes genes are another group of genes that maintain a proliferative state of neural progenitors (Kageyama et al., 2008a). Similarly, we studied the expression of *Hes1*, *Hes3* and *Hes5* in the midbrain region. No any changes in *Hes5* expression was observed in the ventral midbrain, whereas expression of *Hes1* had decreased (see Fig.9 in II and Fig.1 in III). A decreased level of *Hes1* likely leads to the upregulation of proneural genes (Kageyama et al., 2009). Indeed, in the *Fgfr1^{cko};Fgfr2^{cko}* mutants genes indicating neurogenic fate, like *Ngn2*, *Mash1*, *Tis21*, *Jagged* and *Dll1*, and proteins indicating recently differentiated cells, like p57, were upregulated (Fig. 8B). Thus, downregulation of *Hes1* in the *Fgfr1^{cko};Fgfr2^{cko}* mutants could explain the upregulation of proneural genes and, consequently, premature neurogenesis. Although, midbrain-r1 specific expression of *Fgf15* is required for cell cycle exit and proper neural differentiation, increased *Fgf15* levels do not explain the premature differentiation in the *Fgfr1^{cko};Fgfr2^{cko}* mutants (Fig. 9A and A'). Instead, *Fgf15* expression is downregulated or almost lost in the *Fgfr1^{cko};Fgfr2^{cko}* mutants at E11.5. Thus, it is likely that Fgf15 also is regulated by an Fgf-signalling-based feedback mechanism.

In the forebrain, inactivation of *Fgfr1* and *Fgfr2* causes loss of the ventral progenitors (Gutin et al., 2006). In turn, loss of *Fgfr1* and *Fgfr3* in the forebrain causes altered patterning and neural differentiation in the ventro-lateral cell population, and deletion of all three *Fgfr* causes loss of almost the whole telencephalon (Gutin et al., 2006, Paek et al., 2009). Although not all the ventral precursors are lost in the ventral midbrain of the *Fgfr1^{cko};Fgfr2^{cko}* mutants, a similar cooperation of *Fgfs* can, thus, be seen in the maintenance of the neural progenitors both in the forebrain and ventral midbrain.

SoxB1 factors promote a proliferative state of neural progenitors (Pevny and Placzek, 2005). Moreover, the *Sox2* promoter region includes binding sites for Wnt and Fgf regulated transcription factors (Takemoto et al., 2006). In the chick spinal cord, overexpression of *Sox3* prevents neurogenesis, and this defect cannot be rescued by overexpression of *Ngn2* (Holmberg et al., 2008). Thus, at least partly Sox3 acts without Notch-dependent Hes activation. Interestingly, Sox3 has a capacity to prevent activation of neuronal genes by competing for binding sites with Sox11 (Bergsland et al., 2011). Sox11 is involved in the activation of neurogenesis and induction of neuronal markers (Wegner, 2011). This mechanism might explain increased neurogenesis in the ventro-lateral midbrain also in the *Fgfr1^{cko};Fgfr2^{cko}* mutants. Expression levels of Sox11 in the ventral midbrain remain to be elucidated. However, *Sox3^{null}* mutants are viable (Rizzoti et al., 2004) and lack major defects in the maintenance of the neural progenitors. In addition, in *Sox3^{null};Fgfr1* ^{hypomorphs} mutants the mDA neurons develop approximately normally and no major alterations in the thickness of proliferative or differentiated cell layers were observed (unpublished results P. Peltopuro and J. Partanen). Thus, loss of *Sox3* may not be enough to explain the upregulation of proneural genes, increased cell cycle exit and decreased number of the proliferative progenitors. Sox3 may, nevertheless, be required for the maintenance of the neural progenitors cooperatively with other factors. Another Fgf regulated factor promoting proliferation of neural progenitors is *Hes1* (Sato et al., 2010). Fgf signalling regulates Erk activation levels through (Frs2α) and, thus, promotes proliferation of neural progenitors. Moreover, blocking the function of Frs2α causes decreased levels of *Hes1*, and exogenous *Hes1* cannot increase mitoses adjacent to the apical surface if Fgf signalling (or function of Frs2α) is abolished (Sato et al., 2010). Thus, the mitogenic function of *Hes1* seems to be dependent on Fgf signalling. In addition, our results demonstrate that *Hes1* expression also requires Fgf signalling. Thus, loss of *Hes1* and Sox3 in the *Fgfr1^{cko};Fgfr2^{cko}* mutants may both contribute to upregulation on proneural genes and increased differentiation.

5.2.2. Fgf signalling maintains the proliferative state of neural progenitors cell-autonomously (III)

Although, *Fgf8* is expressed strictly in the IsO, the Fgf proteins are able to diffuse further from the expression source. In these more distant locations, the effects of Fgf signalling are limited by the expression of Fgf receptors. Fgf signalling may affect progenitor properties either directly or indirectly. Direct effects can be observed within the cellular population were Fgf signals are received, whereas indirect effect could spread broader and also affect cells which do not express either Fgf ligand or Fgf receptor. To clarify whether Fgf signalling regulates the balance between proliferation versus differentiation directly in a cell-autonomous manner, we studied chimeric embryos in which wild-type cells and the *Fgfr1^{cko};Fgfr2^{cko}* mutant cells formed cell clusters in the ventral midbrain. Interestingly, wild-type tissue maintained the thickness of the Sox2-positive proliferating layer whereas the *Fgfr1^{cko};Fgfr2^{cko}* mutant cell clusters showed a thickened layer of differentiated cells and increased p57 expression indicating increased neurogenic fate inside mutant cell clusters (see Fig. 2 in III). These findings were verified by observations in Shh-Cre induced *Fgfr1^{cko};Fgfr2^{cko}* mutants (see Suppl. Fig. 2 in III). In these mutants, wild-type and mutant tissue formed clusters due to incomplete recombination. As in chimeras, the *Fgfr1^{cko};Fgfr2^{cko}* mutant tissue showed increased neurogenesis. Although wild-type tissue between mutant cell clusters appeared to mediate Fgf signalling normally, it was not enough to rescue the premature neurogenic fate in the

Fgfr1^{cko};Fgfr2^{cko} mutant cell clusters. Therefore, we conclude that in the ventral midbrain Fgf signalling regulates proliferative properties of neural progenitors cell-autonomously.

5.2.3. Normal cell-cell contacts, apico-basal polarity and positioning of the mitotic spindle in the Fgfr mutants (III)

The thinner ventricular zone in the *Fgfr1^{cko};Fgfr2^{cko}* mutants might imply a defect in the maintenance of pseudostratified neural epithelium. Therefore, we wanted to clarify whether a failure in apico-basal polarity or cellular architecture might cause the premature differentiation in the *Fgfr1^{cko};Fgfr2^{cko}* mutants. Apical constituents and cell-cell contacts, such as adherent junctions, and their molecular markers Par3, aPKCλ, Zo1, β-catenin were normally expressed in the *Fgfr1^{cko};Fgfr2^{cko}* mutants (see Fig.4 in III). Normal apical structures such as primary cilia and adherent junctions could also be observed in the mutant ventral midbrain by transmission electron microscopy (see Suppl. Fig. 5 in III). Normal electron-dense areas and tubular structures were identified in the *Fgfr1^{cko};Fgfr2^{cko}* mutant primary cilia and midbodies indicating unaltered microtubule construction at least in these apical regions (see Fig. 5 in III). In addition to apical structures, basal processes visualized by Nestin and their connection to the basal lamina were not abolished in the *Fgfr1^{cko};Fgfr2^{cko}* mutants (see Fig. 5 in III). The orientation of the mitotic spindle is crucial for equal distribution of factors supporting proliferative or neuronal fate. Thus, we measured the division angle of neural progenitors in the wild-types and the *Fgfr1^{cko};Fgfr2^{cko}* mutants. Based on statistical similarity analysis the division angles between wild-type and mutant did not differ significantly (see Fig. 4 in III).

In the *Fgfr1^{cko};Fgfr2^{cko}* mutants, alterations in cell dynamics, such as disassembly of primary cilia in G2 and re-establishment in G1 or cleavage of midbody, could decrease the amount of apical constituents like Par complex proteins, prominin1 and β-catenin and thus cause premature differentiation. Shh and Wnt signalling have shown to be important for cilia structure and primary cilia also act as antennas to receive external Shh, Pdgf, and Wnt signals from the ventricular fluid (Schneider et al., 2005, Eggenschwiler and Anderson, 2007, Corbit et al., 2008). Fgf signalling also is suggested to have a role in the establishment of the cilia (Neugebauer et al., 2009). Thus, loss of Fgf signalling could affect the structure of the cilia and alter Shh or Wnt signalling in the ventral midbrain progenitors. However, this is unlikely when *Wnt1, Shh* and their downstream targets are expressed normally in the ventral midbrain (see Fig. 7 in II). A new fascinating cellular structure, the basolateral primary cilium has been recently identified (Wilsch-Brauninger et al., 2012). These basolateral cilia are involved in the delamination of newly formed neural precursors from the apical surface. As a first sign of delamination, the primary cilia are established in the basolateral rather than apical membrane in neurogenic daughters. It would be interesting to study by transmission electron microscopy, whether these basolateral primary cilia are increased in the *Fgfr1^{cko};Fgfr2^{cko}* mutants. Destabilization of the mitotic spindle also might increase neurogenesis. However, division angle changes in destabilization cases have been relatively clear (Sanada and Tsai, 2005, Fish et al., 2006, Roszko et al., 2006). In the *Fgfr1^{cko};Fgfr2^{cko}* mutants, the change between division angles was not statistically significant.

5.2.4. Directionality and gradient of Fgf signalling (III)

In the neural ectoderm, where cells are apico-basally polarized, and have both apical and basal processes, signals received through these surfaces regulate the decisions to re-enter or withdraw from the cell cycle and differentiate. In addition to inheritance of apical constituents, the factors affecting through basal process or inherited with basal contacts have recently shown to be important for maintenance of the proliferative state (Farkas and Huttner, 2008, Konno et al., 2008, Alexandre et al., 2010).

5.2.4.1. Fgf8 localizes to the basal lamina

We wanted to clarify the direction from which Fgf8 enters the neural progenitors to affect the proliferation versus differentiation balance. We could detect Fgf8 protein expression in several regions, such as MHB, forebrain, brachial arch and limb buds, in which *Fgf8* mRNA is also expressed (see Fig 5 and Suppl. Fig. 6 in III). In all these regions, Fgf8 protein co-localised with the basal lamina proteins like Laminin (see Fig. 5 in III). In cells, that express *Fgf8*, protein was also detected in the apical locations. However, the strong localization in the basement membrane suggests, that Fgf8 is transported to the basal lamina, where it forms an Fgf8 protein gradient decreasing from the source of expression. Indeed, the Fgf target genes are expressed in the regions which correspond to the Fgf8 gradient (see Fig. 5 and Suppl. Fig. 6 in III). The strongest target gene expression could be detected in *Fgf8* expressing cells, but cells outside the *Fgf8* expressing region also show target gene expression at a level decreasing simultaneously with the Fgf8 protein level. Target gene expression was also apparent in mesenchymal cells on the opposing side of the basement membrane (see Fig. 5 in III). Localization of Fgf8 into the basement membrane brings Fgf signals available also for mesenchymal cells

Strong Fgf8 protein signal in the basement membrane might be caused either by basal localization of the Fgf8 protein or fact that Fgf8 protein remains more stable, when it is attached to a basal membrane. Our data suggested that Fgf8 was distributed along the basement membrane, where it formed a concentration gradient. The antero-posterior concentration gradient formed by Fgf8 has been reported previously, although the apico-basal localization of Fgf8 protein has remained unclear (Chen et al., 2009, Toyoda et al., 2010). Our Fgf8 localization seemed to correspond to the immunofluorescence pictures of Fgf8 in the forebrain (Toyoda et al., 2010).Thus, Fgf8 seems to enter the neural progenitor cell from basal side through the basal process. The other explanation for the strong Fgf8 signal in the basal lamina might be its binding to molecules expressed in the basal lamina. The connections between basal lamina molecules and Fgf8 might stabilize Fgf8 protein. However, the Fgf8 would be able to attach to the basal lamina proteins and, thus, remain more stable, downstream signalling is most likely activated in regions where the ligand is available. Indeed, neuronal progenitors cultured in the presence of Laminin and Fgf2 retained expression of a proliferative marker gene, *Sox2*, better than cells growth without these factors (see Fig. 6 in III). This supports assumption that Fgfs combined with basal membrane molecule, Laminin, promotes proliferative state. Moreover, in the late G1 phase when the decision between cell cycle exit and re-entry is made, the nuclei are located on the basal side of the ventricular zone. Basally received Fgf signalling might, thus, modulate the expression of factors, such as *Hes1*, which affect cell fate decisions.

Although, the Fgf signalling has shown to be a major inducer of Erk phosphorylation (Corson et al., 2003), the levels of pErk remained unchanged in the *Fgfr1^{cko};Fgfr2^{cko}* mutant cell bodies and basal processes (see Fig. 5 and Suppl. Fig. 6 in III). Strong Erk activation needs high Fgf8b concentration, which induces cerebellar fate in the isthmus region (Sato et al., 2004), although Erk activation has to be downregulated by Sprouty2 (Suzuki-Hirano et al., 2010). In the midbrain, the Erk activation caused by Fgf signalling might be relatively low and morphogenetic changes might be mediated through some other downstream signalling cascade. Thus, deletion of Fgf signalling does not affect levels of pErk. Instead some other tyrosine kinase receptors may activate or maintain Erk phosphorylation at normal levels in the *Fgfr1^{cko};Fgfr2^{cko}* mutants. However, a recently published study using Fgf8 hypomorphs showed that decreased levels of Fgf8 caused lack of Erk phosphorylation (Crespo-Enriquez et al., 2012). In this system, Erk phosphorylation is strongly linked to Fgf8 expression in the midbrain. Differences in these experimental set-ups might explain the changes in the Erk phosphorylation levels.

5.2.4.2. *Fgf signalling maintains symmetrical proliferative divisions in the ventral midbrain progenitors (III)*

Interestingly, the inner-most layer of the basal lamina, which contains type IV collagen fibers, also is rich in Heparan sulphate- (Yurchenco and Schittny, 1990). Heparan sulphates promote Fgf ligand binding and Fgfr dimerization (Guillemot and Zimmer, 2011). In addition, they are involved in Fgf protein transport (Mason, 2007, Guillemot and Zimmer, 2011, Sunmonu et al., 2011b). Heparin and HSPGs stabilize Fgf-Fgfr signalling complexes (Ornitz, 2000). Moreover, the pair cell assay with midbrain neural progenitors revealed that lack of Fgf2 (basic Fgf) and heparin from cell cultures shifted types of cell divisions from an even distribution of symmetric proliferative, asymmetric self-renewal and symmetric neurogenic to a increased proportion of symmetric, neurogenic divisions (72% of all divisions, see Fig. 6 in III). It is likely that HSPGs stabilize Fgf8-Fgfr signalling complex and promotes transduction of Fgf8 signals the basal side of the neural ectoderm. Thus, Fgf8 might function as a factor that supports symmetric proliferative divisions, and this inductive signal is mediated to neural progenitor cells through the basal surface.
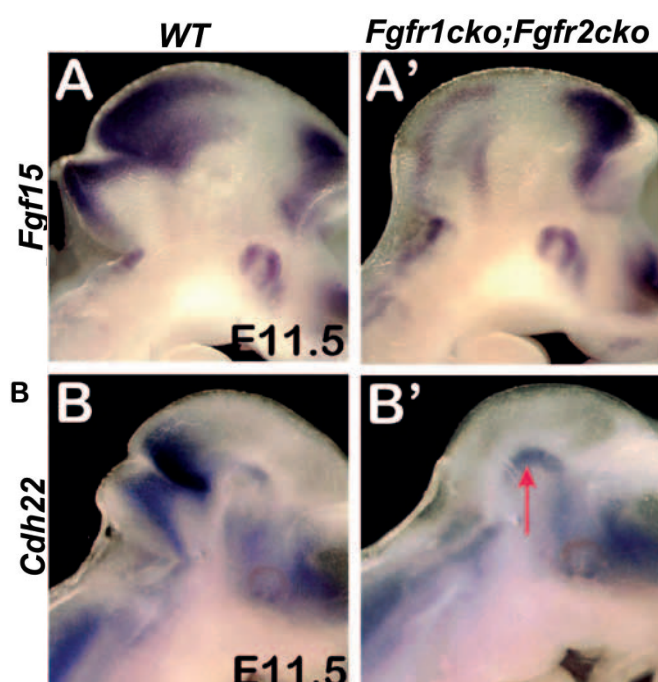
Figure 9. ***Fgfr15*** **and** ***Cdh22*** **are regulated by Fgf signalling**. In the wild type controls *Fgf15* is expressed in the midbrain and rhombomere 1 at E11.5 **(A)**. The most of *Fgf15* expression is lost in the *Fgfr1^{cko};Fgfr2^{cko}* mutants **(A')**. *Cdh22* is strongly expressed in the midbrain-hindbrain boundary at E11.5 **(B)**. In the *Fgfr1^{cko};Fgfr2^{cko}* mutants, the midbrain-hindbrain boundary specific expression is lost **(B')**. Red arrow indicates *Cdh22* expression, which still remains in the ventral midbrain.

5.3. Cadherin-22, a Fgf-regulated adhesion molecule, is not required for maintenance of the Isthmic Organizer (IV)

In addition to neuronal differentiation, Fgf signalling appears to regulate adhesion properties in the MHB. Indeed, the dorsal expression of *Cdh22* and also *Cdh11* were lost in *Fgfr1^{cko}*s (our unpublished results T. Jukkola and J.Partanen, Trokovic et al., 2003). From the *Fgfr1^{cko};Fgfr2^{cko}* mutant embryos, the MHB specific expression of *Cdh22* was lost (Fig. 9B and B'). Thus, we wanted to study expression patterns of several type II cadherins in the developing CNS, especially in the MHB region. Moreover, we wanted to elucidate, whether *Cdh22* is the adhesion molecule crucial for the formation of the compartment boundary between the developing midbrain and r1.

5.3.1. Expression of Cadherin-6, -8, -11 and -22 during brain development

Expression of *Cdh22* starts at E8.0 in the MHB. Expression becomes restricted to a narrow boundary cell population at E10.5 (Fig. 10A). At E11.5, *Cdh22* forms a concentration gradient anterior and posterior from the MHB. Weak expression domains can be detected also in the ventral midbrain and near another secondary organizer region, ZLI (see Fig.1,3 in IV). *Cdh11*, also downregulated in the *Fgfr1^{cko}* mutants, is another cadherin expressed in the MHB and ZLI (see Fig.1,3 in IV). Thus, we wanted to study the expression of these genes more closely near these boundary regions. Two other type II cadherins, *Cdh6* and *Cdh8*, were found to be associated with *Cdh11* in previous studies at later stages (Korematsu and Redies, 1997, Suzuki et al., 1997, Korematsu et al., 1998, Inoue et al., 2008). However, detailed expression analysis of early embryos has not been performed and, thus, we included also *Cdh6* and *Cdh8* into these experiments. Interestingly, *Cdh22* formed a clear anterior-posterior gradient in the midbrain and was detected both in the ventricular zone and the mantle zone at E10.5 (Fig. 10A and B). However, expression was concentrated to lateral regions and was absent from the floor plate cells. In contrast to *Cdh22* expression, *Cdh11* was expressed evenly from the anterior to the posterior region of the midbrain, but was absent from the floor plate cells ((Fig. 10A and B). *Cdh6* was expressed throughout the ventral midbrain, whereas *Cdh8* was not detected in the ventral MHB, but was weakly expressed in the anterior and dorsal regions (Fig. 10A and B). Different type II cadherins seem to be involved in the specification of cellular populations. From E11.5 onwards, these four cadherins were expressed in separate cell populations in the ventral midbrain and diencephalon (Fig. 10A and B, see also Fig 2-5 in IV). At E12.5, *Cdh22* was not expressed in the ZLI region in contrast to *Cdh11*, *Cdh6* and *Cdh8* (see Fig 2 in IV). Thus, *Cdh22* does not seem to play a role in the specification of the ZLI region. These findings suggest that it is likely that some type II cadherins are involved in the formation of coherent MHB before E10.5. After that the expression of different cadherins concentrates in specific cellular populations and then they possibly regulate segregation of brain nuclei. Interestingly, although the MHB specific expression of *Cdh22* was lost in the *Fgfr1^{cko};Fgfr2^{cko}* mutants at E11.5, the ventral expression domain in the midbrain still remained. This finding suggests that the MHB specific *Cdh22* expression is Fgf-regulated, but *Cdh22* expression in the ventral midbrain appears Fgf independent. At birth, *Cdh22* and *Cdh11* are not expressed in overlapping patterns but rather in adjacent cell populations in the midbrain and anterior hindbrain. Thus, they may be involved in the segregation of brain nuclei or specific cell types (see Fig 4. in IV). For example, *Cdh22* is expressed in SNpc and *Cdh11* in SNpr.
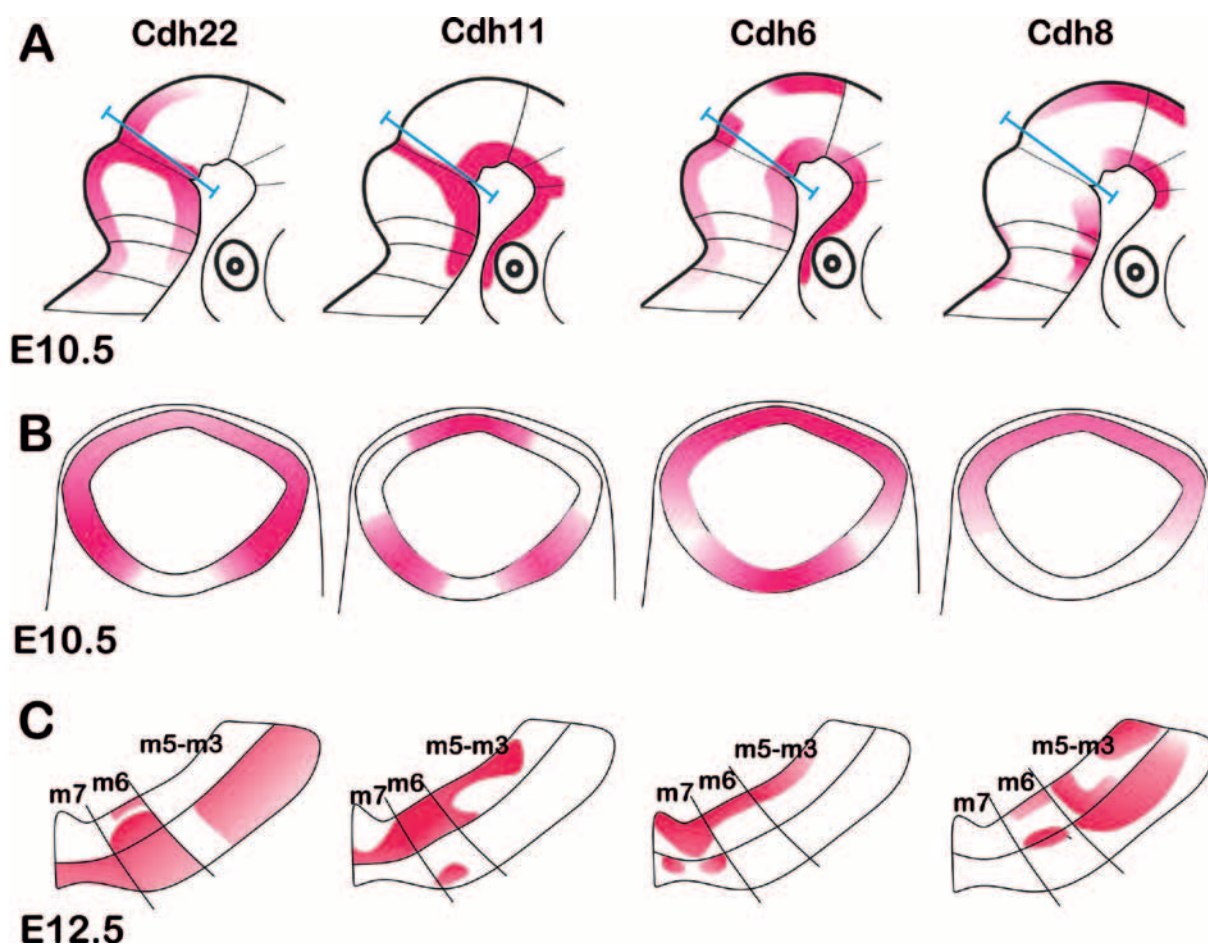
Figure 10. **Dynamic expression patterns of *Cdh22*, *Cdh11*, *Cdh6* and *Cdh8***. Schematic sagittal views of midbrain-hindbrain boundary specific expression domains of *Cdh22*, *Cdh11*, *Cdh6* and Cdh8 at E10.5 (A). Coronal views show dorso-ventral expression patterns of Cdh22, Cdh11, Cdh6 and *Cdh8* near the midbrain-hindbrain boundary (B). At E12.5, these *Cadherins* are expressed in distinct domains in the ventral midbrain.

Similarly in the SC, *Cdh22* is expressed in the superficial layer and the deep layer, whereas *Cdh11* appears in the supraficial layer. The same segregation can be observed in the forebrain. In general, *Cdh22* expression is more concentrated in the ventral regions, such as the preoptic region, hypothalamus and amygdala (see Fig. 5 in IV). *Cdh11* is expressed in more dorsal areas, such as the cerebral cortex, thalamic nuclei and zona inserta. *Cdh22* is expressed in some dorsal brain nuclei, such as the indesium griseum, bed nucleus of stria terminalis, ventro-lateral geniculate nucleus and anterior pretectal nucleus, where it also overlaps with *Cdh11* expression (see Fig. 5 in IV). The only exception of this co-localisation is habenuclear nucleus in which *Cdh22* is strongly expressed in the medial portion and *Cdh11* in the lateral portion.

In summary, at early embryonic stages, *Cdh22*, *Cdh11*, *Cdh6* and *Cdh8* are expressed in restricted and dynamic patterns (Fig.10). At birth, their expression is detected in specific brain nuclei. All these cadherins show unique expression territories. Because classical cadherins prefer homophilic binding, cells expressing the same cadherin favour aggregation with each other. Thus, it is likely that these cadherins are involved in the segregation of distinct brain nuclei and neuronal circuits.

5.3.2. Inactivation of Cdh22 reduces postnatal survival rate

To elucidate the function of Cdh22 during brain development and more closely in the formation of the MHB, we created $Cdh22^{-/-}$ ($Cdh22^{null}$) mutant mice (Turakainen et al., 2009). The $Cdh22^{null}$ mutant embryos did not show major abnormalities between E10.5 and E18.5 (see Fig 7-9 in IV). However, viability of the $Cdh22^{null}$ mutants was decreased after birth and the amount of surviving adult $Cdh22^{null}$ mutants was lower than expected (see Table 1 in IV). The reason for decreased viability remained unknown. Although the post-natal viability of the $Cdh22^{null}$ mutants was decreased, mice that survived did not show any obvious behavioural problems or large alterations in the brain morphology. In addition, the fertility of mice was normal. However, the adult might have some more subtle defects in CNS function. The investigation of these possible symptoms would require more detailed anatomical, physiological and behavioural analyses.

5.3.3. No changes in neural patterning in the Cadherin22 null mutants

We analysed the brain morphology of the embryonic $Cdh22^{null}$ mutants. At E10.5, the structure of the MHB appeared to be unchanged. The midbrain-r1 genes Fgf8, Wnt1, Otx2 and p21 were normally expressed (see Fig 7 in IV). Similarly, whole mount immunohistochemistry revealed a normal appearance of the III and the IV cranial nerves.

Although, the early embryogenesis and formation of the MHB occurred normally in the $Cdh22^{null}$ mutants, some failures might still later appear in brain nuclei that express *Cdh22*. The main neuronal populations in the midbrain and anterior hindbrain were observed at approximately normal locations in the $Cdh22^{null}$ mutants (see Fig. 8 in IV). All main midbrain GABAergic neuron populations, such as the dorsal SC and IC related, the middle midbrain reticular formation and peri-aqueductal gray related and the ventral SNpr related GABAergic neurons, were also present in the $Cdh22^{null}$ mutants. Similarly, the dorsal SC and IC related and medial red nucleus related glutamatergic neurons as well as the SN and the VTA related dopaminergic neurons and the dorsal raphe related serotonergic neurons appeared normal. Moreover, we could not observe major defects in either noradrenergic, glutamatergic, GABAergic neurons or glial structures in the $Cdh22^{null}$ mutant forebrain (see Fig. 9 in IV).

The overlapping expression patterns in at least *Cdh22*, *Cdh11* and *Cdh6* in the MHB suggest redundant function of these cadherins in the maintenance of coherent boundary properties during early embryogenesis. Cadherins are involved in the separation of cell populations inside certain brain compartments (Halbleib and Nelson, 2006). Among large group of type II cadherins, some other cadherins also might be expressed in the MHB, as expression patterns of most type II cadherins are not very well characterized during early neuronal development. In addition, some other cell adhesion or cell guidance molecules, such Ephrins, might be involved in the maintenance of the compartment boundary between the midbrain and the hindbrain (Lee et al., 2008, Lee et al., 2009).

The loss of function mutations in *Cdh11*, *Cdh6* and *Cdh8* have been generated earlier. The $Cdh11^{null}$ mutants show alterations in the hippocampal synaptic connections and behavioural abnormalities in adults (Manabe et al., 2000), but no alterations in the CNS are reported dur-

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ing embryogenesis. Also, *Cdh6^{null}* mice are viable and fertile and they have normal brain morphology, although the cadherin-based cell sorting fails in the cortico-striatal boundary in mutant background tissue cultures (Inoue et al., 2001).

5.3.4. Type II cadherins may cooperate to modulate the coherence of the midbrain-hindbrain boundary

The connection between Fgf signalling and adhesion molecules has been shown in tumour progression, neurite growth and axonal pathfinding (Suyama et al., 2002, Halbleib and Nelson, 2006, Sanchez-Heras et al., 2006). We can see downregulation of *Cdh11* and *Cdh22* at the transcriptional level in the *Fgfr1^{cko}* mutants, but Fgf signalling might also directly or indirectly modulate adhesion properties at several levels: transcriptional, translational, or even at the functional protein level on the cell surface. Interaction at post-translational level occurs between extracellular domains of Fgf receptors and CAM, such as NCAM and Cadherins (Sanchez-Heras et al., 2006). Could Fgf signalling and cadherin cooperate to regulate adhesion properties? Cadherin transcription is regulated either by repressing promoter activity or by methylation (reviewed in Halbleib and Nelson, 2006). Transcriptional repression of *E-Cdh* is regulated by Snail and Slug proteins, which in turn are regulated by Fgf signalling. N and E-Cdhs include an exceptionally large second intron sequence, which at least in E-Cdh contains regulatory elements allowing the modulation of expression levels and pattern in different tissues during development. *Cdh22* does not contain a large second intron; however, the first intron is almost half of the length of the gene (Fig. 6 in IV). This might allow the spatio-temporal regulation of *Cdh22* at the transcriptional level.

Regulation of cadherin-associated adhesion has been suggested to involve the function of catenins when cadherins are regulated by an inside-out based mechanism, similar to integrins (Gumbiner, 2005). In integrins, intracellular binding to Talin causes conformational changes in the extracellular domains of integrins, which increases their affinity to ECM molecules and stabilizes the adhesive properties (Gumbiner, 2005). Similarly, catenins might change the binding properties to the actin-cytoskeleton by regulating signalling molecules, that modify the actin cytoskeleton, or more directly changing the binding affinities of the cadherin extracellular domain, likely by decreasing clustering or dimerization. The mechanism how the catenins regulate cadherin extracellular domain remains to be elucidated (Gumbiner, 2005). Posttranslational regulation of cadherins involves to exocytosis or transportation to the cell surface and turnover of cell-surface-associated cadherins (reviewed in Halbleib and Nelson, 2006). The exocytosis of cadherins is dependent on β-catenin. Cadherin levels in the cell surface are regulated by endocytosis. Tyrosine kinases have been suggested to be involved in the modulation of β-catenin phosphorylation and consequently cell adhesion properties (Lilien et al., 2002). Canonical-Wnt and Fgf-dependent PI3K-Akt signalling are involved in the regulation of β-catenin and Snail through Gsk3β in cancer cells (Katoh and Katoh, 2006), and might thus be involved in the modulation of cell adhesion during neurogenesis. Whether and how Fgf signalling is involved in the regulation of Cadherins would be interesting to study.

Although the *Cdh22^{null}* mutants showed normal MHB development, among the large family of type II cadherins might be other cadherins, such as *Cdh6* or *Cdh11*, which are redundantly expressed with *Cdh22* during boundary formation and maintenance and function cooperatively, probably through cis-based clustering, in the MHB cells. Fgf signalling may also regulate their function. Generally, the loss of function phenotypes of Cdhs have been relatively mild

compare to their expression patterns, thus specific cocktail of distinct cadherins is likely needed for specification of certain neuronal population. Clarifying the role of cell-cell adhesion at the MHB would be to require simultaneous deletion of several members of this cadherin subfamily. The other option would use cell or tissue culture methods to reveal for example the cell segregation in separate cell populations as was shown in the *Cdh6^{null}* mutants (Inoue et al., 2001).

Whether and how Fgf signalling regulates adhesion properties in the midbrain-hindbrain boundary remains to be elucidated. However, studies with *Drosophila* suggest that a functional interaction between Fgfr and cell adhesion molecules is evolutionary conserved (Garcia-Alonso et al., 2000, Forni et al., 2004) and, thus, is probably essential for pivotal cellular functions. Understanding the mechanisms how Fgfr and cell adhesion molecules interact, how this interaction is regulated and in which process this interaction is involved brings new tools for studying the formation of compact cell populations and, probably, preventing tumor progression.

CONCLUDING REMARKS

The importance of the midbrain-hindbrain boundary as an organizer region was discovered two decades ago, and Fgf8 had been recognized as an organizer molecule (Martinez et al., 1991, Crossley et al., 1996). Fgf8 has an ability to induce both specific structural characteristics and a midbrain-hindbrain boundary gene expression profile. Furthermore, inactivation of *Fgf8* in the mouse midbrain-hindbrain boundary results in large deletions, especially in the dorsal regions, mainly caused by programmed cell death (Chi et al., 2003). *Fgfr1* is the only Fgf receptor robustly expressed throughout the midbrain and anterior hindbrain, and, therefore has been suggested to be the primary mediator of Fgf8 signals. However, in contrast to the *Fgf8* mutants, the phenotype of the *Fgfr1* mutants is relatively mild (Trokovic et al., 2003). Interestingly, the coherence of the midbrain-rhombomere1 boundary was disturbed and cells appeared to mix across the midbrain-hindbrain boundary. This revealed a slowly proliferating boundary cell population at the midbrain-hindbrain border, which is lost in the *Fgfr1* mutants. However, in the *Fgfr1* mutants some Fgf signalling was still mediated suggesting a redundant function with other Fgf receptors.

In addition to *Fgfr1*, two other *Fgfr2* and *Fgfr3* are expressed in the midbrain-r1 territory. This study showed that neither of these alone is required for the development of the midbrain and anterior hindbrain. However, further studies revealed cooperative roles for Fgfrs in the development of the midbrain and anterior hindbrain, Fgfr1 being the primary mediator of the Fgf signals. Fgf signalling is required for cell survival, normal antero-posterior patterning and maintenance of the neural progenitor cells. Inactivation of Fgf signalling caused the loss of several neuronal populations, such as dopaminergic neurons, a thinner proliferative cell layer, and premature neurogenesis. Loss of Fgf signalling led to a downregulation of proliferative factors, Sox3 and *Hes1*, which in turn induced sustained expression of proneural genes and premature neurogenesis. Moreover, we localized Fgf8 protein expression to the basal lamina. From there it may act as a proliferative signal, which cells receive through the basal process.

In addition to Fgfr cooperation, we studied molecules possibly involved in the regulation of cell adhesion properties in the specific midbrain-hindbrain boundary cells. We generated a knock-out allele of a midbrain-hindbrain boundary specific, Fgf-regulated adhesion molecule, *Cdh22*. However the *Cdh22* mutants did not showed any patterning defects in the midbrain and anterior hindbrain or other brain nuclei. Thus, *Cdh22* alone is not required for maintenance of the cell adhesion properties in the midbrain-hindbrain boundary or development of neuronal populations during embryogenesis. Cadherins, as a large group of cell adhesion molecules, likely act redundantly to maintain cell adhesion properties in the midbrain-hindbrain boundary. The simultaneous inactivation of distinct cadherins may be required to achieve better understanding whether and how cadherins regulate coherence of the compartment boundary in the midbrain-hindbrain border.

Although inactivation of *Fgf8* from the region further elucidated the importance of Fgf signalling in the midbrain-r1, our experiments with different inactivated *Fgfr* alleles and their combinations allowed a more detailed analysis Fgf signalling-dependent functions in different compartments of the midbrain and anterior hindbrain. These studies revealed differences in the vulnerability of neuronal populations to loss of Fgf signalling.

*FGF*s are associated in several medical disorders. *FGF* signalling and *FGFR* receptor levels are decreased in post-mortem specimens of human patients with major depression (Evans et al., 2004, Riva et al., 2005, Guillemot and Zimmer, 2011). Thus, dysfunction of the *FGF* signalling pathway in the cortical regions and hippocampus likely predisposes patients to psychiatric diseases. Although, our studies show how Fgfs regulate the early development of dopaminergic neurons, the role of Fgf signalling in adults, especially in the maintenance of dopaminergic neurons, remains to be elucidated. Interestingly, allelic variation of the Fgf signalling molecule, *FGF20,* has been associated with Parkinson's disease in humans (van der Walt et al., 2004). In vitro studies have suggested neuroprotective role for Fgf20 specifically in the dopaminergic cell lineages (Ohmachi et al., 2000, Ohmachi et al., 2003, Murase and McKay, 2006). In contrast, a Parkinson's disease risk allele of *FGF20* is linked to stabilized expression of *FGF20* (Wang et al., 2008). Furthermore, Fgfs are able to upregulate expression of α-synuclein, which is able to accumulate into Lewy bodies (Rideout et al., 2003). These Lewy bodies are typical, abnormal protein aggregates inside neurons in Parkinson's disease. Thus, a role for Fgf signalling in being protective or detrimental for dopaminergic neurons remains unclear and requires further studies. Fgf signals, especially *FGF2*, are associated with neuroprotection in brain injuries (Guillemot and Zimmer, 2011). Thus, Fgf signalling molecules might have therapeutic value in neuronal damage.

Although *FGF*s might function as neuroprotective molecules, the adult mammalian brain has limited capacity to self-renew or self-repair. Thus, in vitro cultured stem cells are valuable tools to gain material for replacement therapies. Fgfs can be used as molecular cues towards certain neuronal lineages. In neural stem cell cultures, Fgfs can also be used to promote stem cell proliferation and prevent differentiation. Our studies suggest that Fgf signalling supports symmetric, proliferative divisions and inhibits symmetric, neurogenic divisions at least in neuronal progenitor cultures from the ventral midbrain. Furthermore, the genetic mechanisms behind differentiation of specific neuronal subtypes offer possibilities to find the molecules that could be used as therapeutics. Basic research of the molecules that promote the specification of distinct cellular identities, can be used to differentiate neural progenitors towards a certain cell lineage. Thus, molecules involved in, for example, early developmental processes of the dopaminergic neurons have recently been of great interest. In vitro cultured stem cells and neural progenitors that are differentiated towards a certain lineage might be tools of stem cell therapies in the future. Although the functions of distinct signalling molecules are relatively well known, the interplay between different signalling cascades creates challenges in the future research. The molecular mechanisms and signalling cascades elucidated by developmental biology studies will give a better understanding of how distinct neuronal cell populations develop and which molecules regulate the generation of neuronal diversity and maintenance of these populations. This knowledge may open doors for finding new, more effective therapeutics for neuronal disorders.

ACKNOWLEDGEMENTS

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