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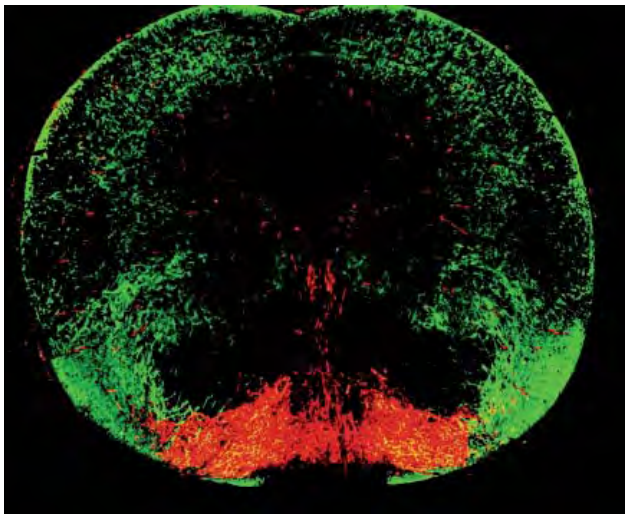
PAULA PELTOPURO

Transcriptional Regulation of GABAergic Neuron Differentiation in the Developing Diencephalon, Midbrain and Anterior Hindbrain



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Transcriptional regulation of GABAergic neuron differentiation in the developing diencephalon, midbrain and anterior hindbrain

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Academic dissertation

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Jokaisen saavutetun kukkulan takana
kohoaa vielä korkeampi ja ihanampi kukkula.
Mitä etsijä yhtenä päivänä luulee totuudeksi,
saattaakin seuraavana päivänä osoittautua vain osaksi sitä.
Salli minun kysyä, voiko ajatella jotain pelottavampaa kuin päätyminen kohtaan,
missä kehitys pysähtyy, paikkaan mihin sinun on jäätävä,
koska et voi mennä edemmäksi ja oppia lisää.

-Axel Fredenholm

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LIST OF ORIGINAL PUBLICATIONS

This Thesis work is based on following original publications, manuscripts and unpublished data. These are referred in the text by their roman numerals.

I. Peltopuro, P., Kala, K. & Partanen, J. 2010, "Distinct requirements for Ascl1 in subpopulations of midbrain GABAergic neurons", *Developmental biology,* vol. 343, no. 1-2, pp. 63-70.

II. Virolainen, S-M., Achim, K., Peltopuro, P., Salminen, M., Partanen, J. "Transcriptional regulatory mechanisms underlying the GABAergic neuron fate and prosomere identity in the developing diencephalon", *Manucript submitted to Development*

III. Achim, K. *, Peltopuro, P.*,. Hui-Hsin, T., Zachariah, A., Åstrand, M., Rowitch, D., Salminen, M., Partanen, J., "*Tal2* and *Tal1* function is essential for specification of mid- and hindbrain GABAergic neuron subtype identity", Manucript

IV. Achim, K., Peltopuro, P., Lahti, L., Li, J., Salminen, M., Partanen, J., " Distinct developmental origins and regulatory mechanisms for GABAergic neurons associated with dopaminergic nuclei in the ventral mesodiencephalic region", *Development* 139, 2360-2370 (2012)
*equal contribution

ABBREVIATIONS

5-HT	5-hydroxytryptamine, serotonin
ANR	Anterior neural ridge
A-P	Antero-Posterior
APN	Anterior Pretectal Nucleus
BrdU	Bromodeoxyuridine
BMP	Bone morphogenic protein
bHLH	basic Helix-loop-Helix transcription family
CGE	Caudal Ganglionic Eminence
CNS	Central Nervous System
DC	Diencephalon
dI	dorsal interneurons
dILa	dorsal interneuron late a
dILb	dorsal interneuron late b
dLGN	dorsal lateral geniculate nucleus
dPAG	dorsal Periaquaductal grey
D-V	Dorso-Ventral
EGF	Epidermal growth factor
EGL	external granular layer
FB	Forebrain
FGF	Fibroblast growth Factor
GAD	glutamic acid decarboxylase enzyme
GABA	Gamma-aminobutyric acid
GABAn	GABAergic neuron
HB	Hindbrain
HD	Homeodomain
HLH	Helix-loop-helix
HMG	High mobility group
IC	Inferior colliculus
IGL	Intergeniculate Leaflet
IHC	Immunohistochemistry
INM	Interkinetic Nuclear Migration
IsO	Istmic Organizer
ISH	In situ hybridisation
IZ	Intermediate Zone
KCC2	potassium-chloride-cotransporter 2
LGE	Lateral Ganglionic Eminence
MB	Midbrain
MBRf	Midbrain Reticular Formation
MGE	Medial Ganglionic Eminence
MHB	Midbrain-hindbrain Boundary
ML	Molecular layer
MZ	Mantle Zone
mRF	midbrain Reticular Formation

Ngn	Neurogenin
NKCC1	Na-K-2Cl cotransporter
NPY	NeuropeptideY
P1	Prosomere 1, pretectum
P2	Prosomere 2, thalamus
P3	Prosomere 3, prethalamus
PAG	Periaquaductal grey
PC	Purkinje cell
Penk1	proenkephalin1
PNS	Peripheral Nervous System
oSVZ	outer Subventricular zone
r1	Rhombomere1
RL	Rhombic lip
SHH	Sonic hedgehog
SC	Superior Colliculus
Six	Sine-oxulis homeodomain transcription family
SNP	Short neural precursor
SN	Substantia Nigra
SNpc	Substantia Nigra pars compacta
SNpr	Substantia Nigra pars reticulata
SVZ	Subventricular zone
TF	Transcription factor
TH	Tyrosine hydroxylase
Tx	Tamoxifen
vLGN	Ventral Lateral geniculate nucleus
vMB	ventral Midbrain
vPAG	ventral Periaquaductal grey
VTA	Ventral tegmental area
VZ	Ventricular Zone
ZLI	Zona limitans Interthalamica

ABSTRACT

The delicate balance of excitation and inhibition of neuronal activity is important for normal brain functions. Gamma-aminobutyric acid, GABA, is the predominant neurotransmitter in the adult brain regulating inhibitory synaptic transmission. GABA is employed in over 40% of the synaptic connections in the central nervous system and GABAergic neurons can be found in all regions of the brain. Mature GABAergic neurons are essential elements of neuronal circuitries by coordinating, balancing and reducing the excitatory activity of neurons. Imbalance between neuronal excitation and inhibition can lead to severe psychiatric and neurological diseases. Several anxiolytic, sedative and addictive drugs act by modulating GABA-mediated inhibitory neurotransmission.

The regulation of GABAergic neuron differentiation has been extensively characterized in the developing forebrain. However, GABAergic neurons are abundant in more posterior brain regions. Although these GABAergic neurons mediate crucially important functions in mature brain and are involved in psychiatric illnesses, rather little is known of the developmental mechanisms of GABAergic neurons in the diencephalon, midbrain and anterior hindbrain.

Neuronal differentiation and identity is regulated by cell-type specific transcription factors. In this study, we have characterized Ascl1 as well as Gata and Tal family transcription factor functions in the GABAergic neuronal precursors. We showed that the proneural gene *Ascl1* was region specifically required for GABAergic neurogenesis in distinct midbrain GABAergic neuron subpopulations. By comparing the GABAergic neuronal phenotypes and specific transcription factor expressions in *Ascl1* and *Gata2* mutant mice, we proposed a model how specific neuroepithelial regions of developing midbrain give rise to different mature GABAergic neuron subpopulations. We also characterized the requirement of Gata2 and Ascl1 for GABAergic neurogenesis in diencephalic prosomeres and showed that there *Gata2* acts as a post-mitotic selector gene and is specifically required for the differentiation of different GABAergic neuron sybtypes in the developing diencephalon. In the midbrain and diencephalon Ascl1 promoted or repressed cell cycle progression depending on the regions of developing neuroepithelium.

We also analyzed the functions of Gata2 cofactors Tal1 and Tal2 for midbrain GABAergic neuron differentiation. Tal2 and Gata2 were both required for complete activation of GABAergic target genes and subsequent GABAergic neuron identity formation in the midbrain. They were not needed in the anterior hindbrain, where Tal1 was required for GABAergic neuron differentiation.

Finally, we studied the developmental origin of midbrain Ventral tegmental area and Substantia Nigra-associated GABAergic neurons and showed that these GABAergic neurons are born in ventrolateral rhombomere 1 and migrate to midbrain as postmitotic precursors. Thus, we showed that there is developmental heterogeneity between different GABAergic neuron subpopulations in the midbrain. Compared to other midbrain GABAergic neuron subpopulations, the GABAergic neurons associated with Ventral tegmental area-Substantia Nigra dopaminergic nuclei utilize genetically different transcriptional regulation and have unique developmental origins.

These studies elucidate the transcriptional regulatory mechanisms controlling the development of essential neuronal populations in brain regions which have important impact for normal brain functions. Understanding the developmental processes and molecular pathways regulating GABAergic neuron differentiation may thus lead to new advanced diagnostics and treatment for psychiatric disease.

1. REVIEW OF THE LITERATURE

1.1 Neuronal architecture and signalling

The central nervous system (CNS) is one of the first organ systems to develop during the embryogenesis and it contains enormous amounts and a wide variety of neuronal and glial cells creating neuronal networks critical for all brain functions. The correct organization, structure and connections of neurons are essential for the nervous system to control our body functions. Functional or structural abnormalities in this system are directly connected to neurological disorders. Neurons are specialized for exchanging information with external and internal environments of the body. They transmit information inside the cell by fast and transient changes in the electrochemical properties of the cell membrane.

A nonstimulated neuron typically has resting potential of -60 mV across the cell membrane, the inside being negative relative to the outside. The electric potential of the cell membrane is created by different ion composition between the cytosol and extracellular fluid. Permeability of the cell membrane for K^+ ions differs when compared to Na^+, Cl^- and Ca^{2+} ions ions, creating concentration differences of these ions between the inside and outside of the cell. In a resting membrane potential at -60mV, there is higher concentration of K^+ ions inside the cell and Na^+, Cl^- and Ca^{2+} ions are actively kept outside the cell. Typical neurons have multiple dendrites which receive chemical signals from axon termini of other neurons. Action potential launches the release of chemical neurotransmitters to the synapse between two neurons and enables the neurotransmitter released from a presynaptic cell to bind its receptor on the postsynaptic cell membrane. Neurotransmitter exocytosis is mediated by a Ca^{2+} influx from the extracellular fluid through voltage gated Ca^{2+} ion channels which are opened as the action potential arrives to the axon termini. Based on the rate of cellular response that a specific neurotransmitter receptor mediates, channels can be divided into two classes. Ligand gated ion channels, which mediate fast postsynaptic responses and G-protein coupled receptors, which mediate slow responses.

Binding of neurotransmitter to the receptor on the post-synaptic cell membrane leads to the opening of transmembrane ion channels and ion flow inside the cell down to its ion concentration gradient. Different neurotransmitters bind to their particular receptors and cause changes in the cell membrane permeability to specific ions. The outcome of ion movement through the postsynaptic cell membrane creates either hyperpolarisation and inhibiton or depolarization and activation of postsynaptic neuron. Glutamate, the principal excitatory neurotransmitter binds to ligand gated ion channels, inducing rapid and direct postsynaptic depolarization which is result of Na^+ and $Ca2^+$ influx to the cell. In contrast, other neurotransmitter such as GABA and glysine hyperpolarize and inhibit the target neurons.

1.2 GABA mediated signalling in the CNS

The balance between inhibition and excitation of neurons in the mature brain is fundamental for normal brain functions. Gamma-aminobutyric acid (GABA) is the princi-

pal inhibitory neurotransmitter in the mature mammalian brain. GABA is synthesized from glutamate in the reaction that is catalyzed by glutamic acid decarboxylase enzyme (GAD) and transferred into neurotransmitter vesicles that are exocytosed from the presynaptic cell Ca^{2+} -dependently. GABA can bind and activate either GABA-A and GABA-C ionotropic receptors which are ligand-gated Cl- ion channels and induce Cl- to enter the cell, causing hyperpolarisation (Bormann, 2000). Binding of GABA to metabotropic G-protein coupled GABA-B receptors activates K+ efflux through K+ channels resulting hyperpolarisation and inhibition of the post synaptic cell. In addition, in the presynaptic axon termini GABA-B receptors inhibit $Ca2^{+}$ currents and neurotransmitter release from the the presynaptic cell (Pinard et al., 2010). Eventually the uptake of GABA by glial cells and nerve terminals stops the neurotransmission.

GABA producing neurons are abundant and can be found in all regions of the CNS. In the forebain and neocortex GABAergic neurons are morphologically diverse and can be classified into subtypes based on their neurochemical composition, synaptic connectivity and projection pattern, location and gene expression (Kawaguchi and Kubota, 1997). Mature GABAergic neurons coordinate, balance, and reduce the excitatory activity of other neurons and therefore are essential for neuronal circuit function. Abnormalities in the balance between excitation and inhibition can lead to severe psychiatric and neurological diseases. Perturbarations in the GABAergic neurotransmission has been suggested to cause anxiety, bipolar disorders, and addiction. In addition, various commercial pharmaceuticals such as anxiolytix and sedative as well as addictive drugs modulate GABA neurotransmission.

1.3 GABA in developing brain

In addition to its function as a neurotransmitter GABA has multiple roles in the proliferation, differentiation, and maturation of neurons into functional neuronal networks during brain development. In contrast to the adult brain, in immature brain GABA is initially an excitatory neurotransmitter depolarizing developing neurons. This is a result of high intracellular concentration of Cl^- ions which are generated by the expression difference between membrane bound Cl-transporters Na-K-Cl cotransporter (NKCC1) and potassium-chloride-cotransporter 2 (KCC2) in immature neurons. NKCC1 predominates in immature neurons and drives Cl^- ions inside the cell. Binding of GABA into GABA-A receptors establishes an efflux of Cl^- ions down to their concentration gradient and depolarization and excitation of immature neurons. On the contrary, in mature neurons KCC2 expression predominates and generates low intracellular Cl^- concentration as it removes Cl^- into the extracellular space. In such conditions, subsequent GABA activation results in Cl^- influx and membrane hyperpolarization (Li et al., 2002;Wang et al., 2003).

The early depolarizing activity of GABA on immature neurons and progenitor cells has been shown to be important for several developmental steps including progenitor cell proliferation and further differentiation. Studies done on neurospheres and brain slices have shown that GABA-receptor activation in proliferative progenitor cells induces an increase in the concentration of intracellular Ca^{2+} and inhibits cell cycle progression(Liu et al., 2005;LoTurco et al., 1995;Nguyen et al., 2003). In addition, GABA can act as

chemoattractant in the cortex for tangentially migrating interneurons and regulate their migration motility concentration dependently(Behar et al., 1996;Behar et al., 2000). These motogenic effects of GABA are associated with the differential expression of KCC2 during development. GABA mediates motogenic activity through GABA-A receptor in immature migrating interneurons expressing low levels of KCC2. Once the migrating interneurons reach the cortex they induce upregulation of KCC2 which act as a stop signal and reduces migration (Bortone and Polleux, 2009).

To become part of neuronal circuit, a developing neuron has to generate a correct morphology and establish and refine synaptic contacts with other neurons in the environment. GABA mediated depolarization of immature neurons also regulates synaptogenesis and neuronal circuit formation. In newborn neurons, the expression of GABA-A receptor predominates compared to the glutamatergic receptors (Tyzio et al., 1999). GABA mediated depolarizations regulate activity dependent glutamatergic synapse and dendritic spine formation in both embryos and adults and balance between excitation and inhibition in developing neuronal circuits (Akerman and Cline, 2007;Ben-Ari et al., 2007;Ge et al., 2007;Huang, 2009;Wang and Kriegstein, 2009).

In summary, GABA has multiple versatile roles during neuronal development starting from regulation of neural progenitor proliferation and differentiation to migration of neurons and maturation of functional neuronal circuits.

1.4 Development of the nervous system

1.4.1 Neural induction

The organization of the central nervous system begins at very early stages of development. The early development of an embryo requires the interplay of signalling molecules secreted by extraembryonic tissues and three embryonic germ layers: endoderm, mesoderm and ectoderm. Central nervous system is specified from the ectoderm, which is induced and fated to generate neural tissue as a result of combination of inductive signals from the adjacent tissues and ectoderm itself. These inductive processes was first discovered in the amphibian embryos in the 1920´s by Spemann and Mangold and which later led to the investigation of soluble growth factors and their inhibitors which regulate the neural plate formation before and during gastrulation. The molecular interplay of signalling molecules such as Fgfs, Wnts and BMPs induce the neural plate cells to form a thickened and flat epithelial-like structure, which expresses a unique pattern of transcription factors and signalling molecules (Wilson and Edlund, 2001). After the development of the neuroepithelium, a process called neurulation results the formation of the neural tube. At the beginning of neurulation cellular rearrangements in the neural plate cells induce bending and elevation of neural folds which fuse together in the dorsal midline creating neural tube (Vieira et al., 2010).

1.4.2 Development of early brain vesicles

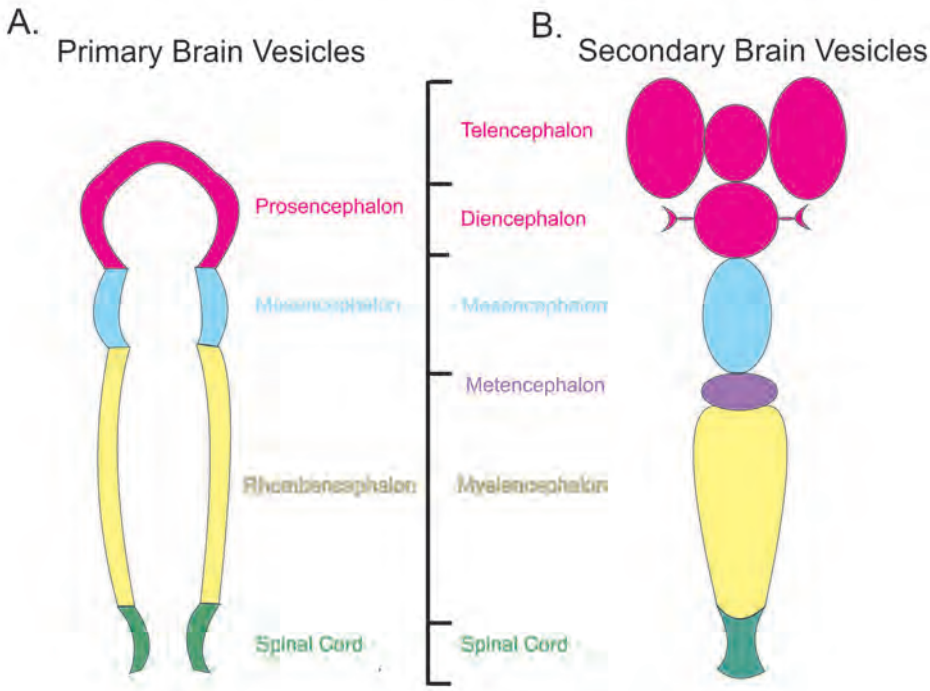
Control of the regional development of the neural tube is guided by signalling molecules secreted from the surrounding tissues and progenitor cells in the wall of developing neural tube. Local environmental signals regulate cell proliferation of progenitor cells which differentiate to form different functional regions of the brain. The early neural tube will form from anterior to posterior the three primary brain vesicles, prosencephalon (forebrain), mesencephalon (midbrain) and rhombencephalon (hindbrain). Regulation of neural tube patterning is largely controlled by morphogenetic signals secreted during early gastrulation.

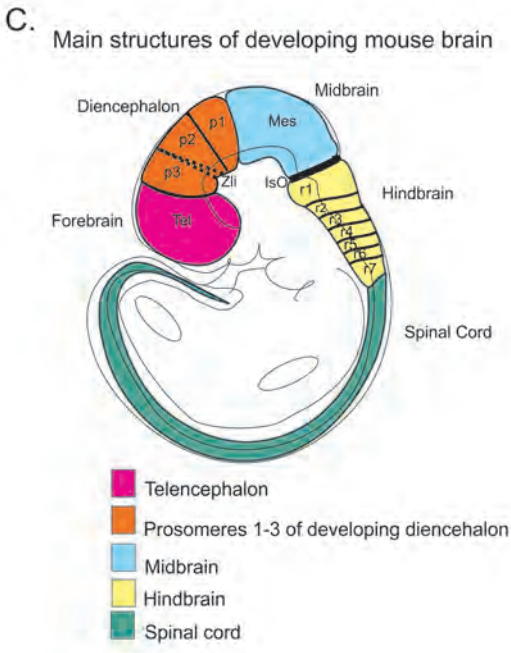
This patterning during neural induction induction is essential for antero-posterior and dorso-ventral regionalization of the early neural tube which is later modified by secondary organizers. The specification of the neural tube begins from the anterior region where the neural tube swells and forms three primary brain vesicles. The most anterior region, prosencephalon is subdivided into paired telencephalon and diencephalon. The posterior region called rhombencephalon becomes divided into metencephalon and myelencephalon which will later in the development form cerebellum and medulla, respectively. Future midbrain is formed from the structure between prosencephalon and rhombencephalon called mesencephalon (Figure 1).

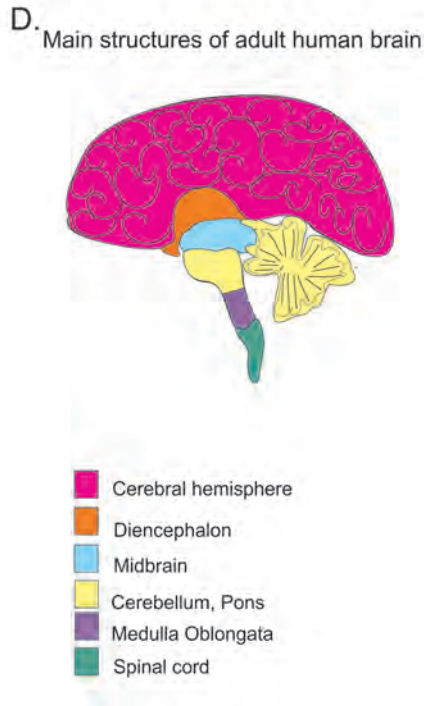
1.4.2.1 Anterior-Posterior patterning of the neural tube

The complex formation of antero-posterior (AP) axis of central nervous system is connected to the early events of gastrulation and neural induction. Morphogenic gradients created in the early neural plate stage guide neuroepithelial cells to adopt specific identities along AP axis of neural tube and differentiate according to this regional identity. The molecular interplay of Fgfs, Wnt and BMP inhibitors is essential for the development of head to tail structures (Vieira et al., 2010). These gradients activate cell specific downstream target genes in the neural progenitors and also create local competence for cells to form secondary organizers which also secrete Fgf, Wnt and Bmp family growth factors. The ability of neural progenitors to later adopt region specific subtype specification depends on these secondary organizers which typically form in the boundary regions separating two developing brain compartments. Secondary organizers are formed from specialized cells that have ability to regulate, refine and guide the regional differentiation of adjacent tissues (Echevarria et al., 2003). There are three well-studied local signal-

Figure 1. The specification of neural tube and the main structures of developing mouse and adult human brain. A. Three vesicle stage neural tube. B. Five vesicle stage neural tube. C. Main structures of developing mouse brain D. Main structures of adult human brain. A. The most anterior prosencephalon gives rise to telencephalon and diencephalon which comprise the forebrain. Mesencephalon forms midbrain and rhombencephalon is divided into metencephalon and myelencephalon which will form future cerebellum and medulla respectively. In A. and B. the colours indicate different brain vesicle structures and their derivatives. In D the colours indicate derivative structures in adult human brain.p1, prosomere 1(pretectum); p2, prosomere 2(thalamus); p3, prosomere 3 (prethalamus); tel, telencephalon; Zli Zona limitans Interthalamica,; IsO, Isthmic Organizer; r1-r7, rhombomeres 1-7.


A.
Primary Brain Vesicles
Prosencephalon
Mesencephalon
Rhombencephalon
Spinal Cord
B.
Secondary Brain Vesicles
Telencephalon
Diencephalon
Mesencephalon
Metencephalon
Myelencephalon
Spinal Cord


C.
Main structures of developing mouse brain
Diencephalon
Midbrain
Mes
p1
p2
p3
Zli
IsO
r1
r2
r3
r4
r5
r6
r7
Forebrain
Tel
Hindbrain
Spinal Cord
Telencephalon
Prosomeres 1-3 of developing diencehalon
Midbrain
Hindbrain
Spinal cord


D.
Main structures of adult human brain
Cerebral hemisphere
Diencephalon
Midbrain
Cerebellum, Pons
Medulla Oblongata
Spinal cord

ling centers in the developing neural tube: Anterior Neural Ridge (ANR), Zona limitans Interthalamica (ZLI) and Ishtmic Organizer (IsO).

1.4.2.2 Antero-Posterior patterning of the forebrain and diencephalon

The most anterior secondary organizer, anterior neural ridge (ANR) is a source of Wnt inhibitors and Fgfs and their graded activity regulates early differentiation of anterior forebrain structures (Houart et al., 2002;Nordstrom et al., 2002). Subdivision orchestration in the CNS is based on the expression of transcription factors that contain homeodomain regions which enable them to recognize and control the expression of target genes. In the forebrain, there are multiple transcription factors that define the development of specific regions. Sine-oculis (Six) homeodomain transcription family is activated in the anterior neural plate, in the area of predominant Wnt inhibition. Six3 actively represses *Wnt* gene transcription and promotes anterior cell fate specification in the anterior presumptive forebrain and diencephalon (Lagutin et al., 2003;Lavado et al., 2008). Another homeodomain transcription factor, Iroquis (Irx) is expressed complementary to Six3 in more caudal region of prospective diencephalon and midbrain. The forebrain is transversally divided into segments; secondary prosencephalon including telencephalon and diencephalon which is subdivided into segments called prosomeres, P1/ pretectum, P2/ thalamus and P3/ prethalamus (Puelles and Rubenstein, 2003;Scholpp and Lumsden, 2010). Zona limithans Intrahalamica (ZLI) acts as a local signalling center which is formed between P2 and P3. Prosomere identities are specified during early neural development by region specific expression of transcription factors. ZLI further regulates patterning of thalamus and prethalamus by secreting Shh, Fgfs and Wnts (Garcia-Lopez et al., 2004;Kiecker and Lumsden, 2004;Scholpp et al., 2006;Scholpp and Lumsden, 2010). Other region specific transcription factors refining prosomere identities include Fez in P3, Irx3 and Otx2 in P2.

1.4.2.3 Antero-Posterior patterning of the midbrain and hindbrain

The complementary expression of homeodomain (HD) transcription factors Otx2 and Gbx2 expression domains separate prospective midbrain and anterior hindbrain structures, respectively (Broccoli et al., 1999;Millet et al., 1999). The restricted expression of *Otx2* and *Gbx2* separates the early epiblast into anterior and posterior regions. *Otx2* is expressed at early stage of embryonic development from anterior forebrain to midbrain-hindbrain boundary (MHB) region. *Gbx2* is expressed on the hindbrain side and the MHB is determined by repressive interactions between Otx2 and Gbx2 (Broccoli et al., 1999;Martinez, 2001;Simeone, 2000).

The local signalling center, Isthmic organizer (IsO), is positioned at the interface of these brain compartments and is crucial for the formation of midbrain and anterior hindbrain structures. Otx2 and Gbx2 are essential for the correct positioning of IsO and create competence for midbrain progenitor cells to respond to other region specific signalling molecules (Puelles et al., 2004;Simeone, 2000). IsO secretes multiple intercellular signalling molecules, such as Wnt1 and Fgf8, which are expressed in the adjacent regions of posterior midbrain and anterior hindbrain, respectively. These control the expression of several homeodomain transcription factors (Nakamura et al., 2005;Prakash and Wurst, 2004)(Liu and Joyner, 2001). Bead and transplantation studies made in chick

first revealed the importance of the expression of Fgf8 and Gbx2 for the patterning of the midbrain and anterior hindbrain (Crossley et al., 1996;Martinez et al., 1999).

The master regulatory effects of IsO on MHB patterning are conducted by Fgf8 inducing *Engrailed1/ Engrailed2 (En1/En2*) and *Gbx2* and concurrently repressing *Otx2* and *Pax6* gene expression. Several loss of function studies made in mice have revealed complex molecular cascades regulating the patterning of midbrain-hindbrain boundary region. In general, regional patterning of different brain compartments is determined by combinatory expression of transcription factors that create positive and negative feedback loops (Liu and Joyner, 2001). Fgf8 secreted from Iso induces the expression of homeobox transcription factors En1 and En2, which further activate Paired Box-family transcription factors Pax2/5 (Nakamura et al., 2005)(Nakamura et al., 2008). On the contrary to Wnt1 and Fgf8 expression, En1/En2 and Pax2/5 are expressed across midbrain-hindbrain boundary (MHB) region (Funahashi et al., 1999;Joyner, 1996;Okafuji et al., 1999;Wassef and Joyner, 1997). In more rostral regions Pax6 expression is repressed by Engrailed creating a diencephalon-midbrain border. Engrailed and Pax2 together with Grg4 negatively and positively regulate the expression of *Pax6* in the midbrain creating a boundary between diencephalon and midbrain (Matsunaga et al., 2000;Sugiyama et al., 2000). IsO induced transcription factors interact with competence factors to induce specific patterns of cellular differentiation. For example, Irx2 specifies cerebellar identity (Matsumoto et al., 2004).

In addition, specification of midbrain and rhombomere 1 is regulated by different levels of Fgf signalling intensity between these developing brain structures. This has been shown by ectopic expression of Fgf8 splice variants Fgf8a and Fgf8b which mediate different consequences in the midbrain and anterior hindbrain development. When ectopically expressed in the prospective chick midbrain, the splice variant Fgf8b repressed *Otx2* expression and subsequently changed the midbrain into cerebellar fate. In contrast, ectopic expression of Fgf8a expands the midbrain (Sato et al., 2001). The structural basis for their ability to differentially regulate midbrain-hindbrain development lies in the N-terminal region of Fgf8b which enables it to bind to its receptor with higher affinity compared to Fgf8a and subsequently promote cerebellum development (Olsen et al., 2006). I addition to this, *Hox* genes regulate the A-P segmentation of the hindbrain and spinal cord through sequential and partially overlapping expression within these structures. Fgf8 from IsO represses HoxA2 expression in the most anterior segment of hindbrain, rhombomere 1 which establishes boundary between rhombomere1 and rhombomere2 (Mason et al., 2000).

1.4.2.4 Dorsal-Ventral patterning of the neural tube

In addition to segmentation of neural tube along its A-P axis, it is polarized along its dorsal-ventral (D-V) axis. D-V domains are delineated by multiple signals coming from immediate environment at early neural plate stage. The D-V patterning is best understood in the spinal cord, but other regions of the CNS seem to use similar mechanisms (Ericson et al., 1995). The most important molecule for D-V patterning is Shh which is secreted from the underlying notochord. Shh induces floor plate, a secondary signalling centre, in the ventral region of the neural tube which subsequently secretes Shh. Similarly, TGF-b superfamily proteins, BMPs secreted from the dorsal ectoderm induce

secondary signalling centre in to the roof plate of the neural tube. The morphogen gradient of Shh from floor plate and counteracting gradient of BMPs from roof plate create different domains from ventral to dorsal axis of neural tube. These domains are ventral floor plate, basal plate, alar plate and roof plate.

In the spinal cord Shh gradient further refines ventral neural progenitors to comprise spatially distinct domains along the D-V axis of the neural tube. Progenitors respond differentially to graded diffusion of Shh by regulating the expression of their target genes which belong to HD and bHLH transcription families (Dessaud et al., 2008). These transcription factors (TFs) can be classified into two classes according their response to Shh: Class I are inhibited and Class II are activated by Shh (Briscoe and Ericson, 1999;Briscoe et al., 2000).

In vitro and *In vivo* studies have revealed the complex interaction of different transcription factors in the coordination of ventral neural tube patterning. *Nkx2.2* (Class II) and *Pax6* (Class I) are expressed in adjacent domains in developing neural tube, Nkx2.2 ventrally and Pax6 dorsally. Their expression is regulated by graded Shh signalling and further refined by cross repressive actions of Class I and Class II proteins on each other (Ericson et al., 1997)(Balaskas et al., 2012). Mutually exclusive expression of Class I and Class II proteins eventually generates progenitor domains p0, p1, p2, pMN and p3 which will establish subsequent neuronal subtypes: V0, V1, V2, MN and V3. Similar signalling mechanisms regulate the patterning of forebrain neuroepithelium into dorsal pallium and ventral subpallium of the telencephalon, which later give rise to cerebral cortex and basal ganglia, respectively. Shh can induce the expression of Nkx2.1 which regulates the regional specification of ventral telencephalon (Sussel et al., 1999). In contrast, Shh can repress expression of Pax6 which is required for correct specification of D-V patterning along entire A-P axis of developing neuroepithelium (Ericson et al., 1995;Ericson et al., 1997;Stoykova et al., 2000).

1.5 Differentiation of neurons in the brain

1.5.1 Cell biology of the neuroepithelium

The mammalian brain is constructed from an enormous number of neurons and glial cells. During the development all the neuronal and glial cells of the mammalian CNS are generated from self-renewing neuroepithelial cells that are located in the ventricular zone (VZ) of neural tube. At the beginning of neurogenesis the neural tube is a one cell layer thick structure, composed of neuroepithelial cells, which have elongated appearance and cytoplasmic contacts with both apical and basal surfaces of the neural tube. VZ has a multilayered appearance which is the result of progenitor cell nuclei movement between the apical and basal side of the VZ during the cell cycle. This movement is called interkinetic nuclear migration (INM). Dividing nuclei of the progenitor cells, the M-phase cells, are located in the apical side of VZ. On the contrary, during G1 the nuclei move towards basal side where in S-phase the DNA synthesis will occur. After DNA replication the nuclei move back to the apical side where cell divisions will take place (Figure 2 A) (Taverna and Huttner, 2010)(Gotz and Huttner, 2005).

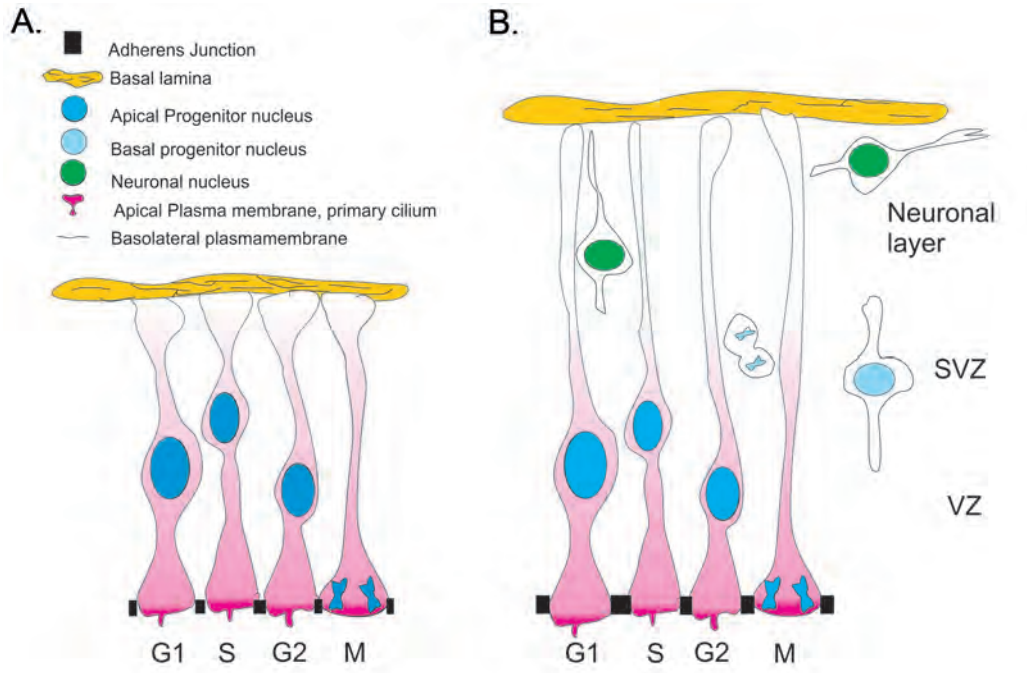


Figure 2. Schematic representation showing apical-basal polarity and internuclear migration of neural progenitors. A. The nuclei of neuroepithelial cells (apical progenitors) move between apical and basal side of the entire ventricular zone (VZ) during the cell cycle. The nuclei are positioned in the basal side during DNA synthesis and they move to apical side (G2) where the cell division (M-phase) takes place. During G1 of the cell cycle, the nuclei move back to basal side. B. The nuclei of radial glial cells of the cortex migrate between apical side of VZ and basal side of subventricular zone (SVZ). Basal progenitors divide by symmetric neurogenic division in the basal side of VZ and form the subventricular zone underlying VZ thereby increasing the number of neurons generated from apical progenitors. There are characteristic intra- and intercellular structures located in specific regions of neuroepithelium. The adherens junctions connect neuroepithelial cells together in the apical side plasmamembrane. Primary cilium of apical plasmamembrane protrudes into ventricle lumen. Trough basolateral plasmamembrane, the neuroepithelial cells are connected to basal lamina. Schematic representation is based on (Taverna and Huttner, 2010).

1.5.2 Neural progenitors in the forebrain

In the cortex, which is derivative of developing telencephalon, neurogenesis is a more complicated process. There progenitor cells were initially categorized into two different cell types, apical and basal progenitors according to their mitotic position in the developing neocortex (Gotz and Huttner, 2005;Kriegstein et al., 2006). The apical progenitors (AP) have epithelial, elongated morphology; they divide on the apical side of VZ and are often referred as neuroepithelial cells (NE) or radial glial cells (RGC) (Gotz and Huttner, 2005;Takahashi et al., 1995). The second progenitor cell type is basal progenitor (BP), also referred to as intermediate progenitor. They are the progeny of apical progenitors

and form the subventricular zone (SVZ) underlying VZ. Basal progenitors undergo symmetric neurogenic divisions in the basal side of VZ thereby increasing the number of neurons generated from apical progenitors (Figure 2 B.). Their morphological characteristics differ from apical progenitors in that they have lost the contact with apical surface and express basal progenitor specific genes like *Tbr2* and *Cox1-2* (Attardo et al., 2008;Haubensak et al., 2004;Sessa et al., 2008). In the cortex the ventricular zone apical progenitors express Pax6 while it is downregulated in the basal progenitors as they start to express Tbr2 in the underlying subventricular zone (Englund et al., 2005).

In recent years the view of simply categorizing cortical progenitors into two different classes has been revised. Diversity in progenitor composition has been proposed to explain the differences in brain size and higher cortical functions between species. Currently, apical and basal progenitors are classified into three different classes, bipolar, monopolar and non-polar, according their cell polarity during mitosis (Fietz et al., 2010). Apical progenitors are predominantly bipolar during M-phase. They maintain contact throughout the cell cycle with both the apical plasma membrane facing the ventricular lumen and with the basal lamina, basally. Another subtype of apical progenitors: short neural precursors (SNP) are monopolar progenitors. They undergo mitosis in the VZ, maintain contact with the apical plasma membrane but retract their basal processes during M-phase (Stancik et al., 2010)(Fietz et al., 2010). The basal progenitors delaminate their apical processes from the apical plasma membrane and undergo mitosis either in the SVZ or in the outer SVZ (oSVZ). Based on the cell polarity during mitosis they can be classified as non-polar basal progenitors, which lack contact with apical membrane, and basal lamina or monopolar oSVZ progenitors which have contact with the basal lamina (Fietz et al., 2010;Fietz and Huttner, 2011;Wang et al., 2011). Compared to the forebrain, less is known about distinct progenitor cell types in other brain regions.

1.5.3 The intracellular control of neural progenitor cell proliferation

The accurate control of the cell number generated during CNS development is crucially important for the correct organisation and function of the mature brain. The amount of cells that will ultimately be generated from progenitor cells is dependent on the developmental stage of the nervous system and the regional position of the progenitor cell in the developing neural tube. In the beginning of neurogenesis progenitors undergo predominantly symmetrical proliferative divisions creating two identical self-renewing progenitor cells. Proliferative divisions are followed by asymmetric divisions whereby a self-renewing progenitor cell produces a copy of itself and a progeny of more differentiated precursors. As development proceeds the neuroepithelium transforms into a multilayered structure where different cell types can be distinguished from each other based on the expression of specific molecular and cellular markers. In different areas of the developing CNS, neuroepithelium transforms into diverse structures as the proliferative cells adopt more differentiated fate.In the midbrain, proliferative progenitors form the VZ and differentiated postmitotic cells the underlying mantle zone (MZ). Postmitotic neurons create connections with other neurons and send projections to other brain compartments creating cell-poor marginal zone underlying mantle zone.

The balance between progenitor cell proliferation and terminal neurogenic differentiation is controlled by several cell intrinsic and extrinsic factors. Cell cycle regulators together with neuronal determination factors have been identified as key determinants in the complex regulation of progenitor expansion versus differentiation (Ohnuma and Harris, 2003). The cell cycle length of G1 phase has been proposed to be an important regulator of neurogenesis. Generally, it is believed that the length of G1 increases towards end of embryogenesis and passing the G1/S checkpoint is pivotal for cell division to occur. Cdk4/CyclinD1 together with Cdk2/CyclinE guides the progression of cell cycle from G1 to S-phase by phophorylating Retinoblastoma protein (Takahashi et al., 1995;Zhang, 1999). The ability of overexpressed Cdk4/CyclinD1 to shorten basal progenitor cell cycle and delay neurogenesis illustrates the importance of the cell cycle length regulation in the progression of neurogenesis (Lange et al., 2009).

Several molecular pathways have been identified in proliferative progenitors which induce the downstream molecular mechanisms that give progenitors ability to self-renew, divide and differentiate to different neuronal subtypes. Some of the well characterized regulators of progenitor identity and maintenance are High mobility group (HMG-box) transcription factors Sox1-3 which comprise the *SoxB1* gene family. Sox2 and Sox3 are expressed at very early stages of embryonic development and are crucial for neuroectoderm and neural fate formation. Signals controlling the early events of neural induction like Fgfs and BMPs are thought to regulate the expression of *SoxB1* genes (Pevny et al., 1998;Rex et al., 1997). Sox1-3 expression is restricted to the proliferative progenitor cells and they maintain the progenitors in an undifferentiated state by suppressing neuronal differentiation in the developing neural tissue (Bylund et al., 2003;Wegner and Stolt, 2005). SoxB1 proteins have more than 90% sequence identity in their DNA binding domain and they have been shown to act redundantly throughout the developing CNS and adulthood by compensating for the loss each other (Episkopou, 2005;Ferri et al., 2004;Pevny and Placzek, 2005). Gain of function studies in chick neural progenitor cells have shown that SoxB1 proteins counteract the effect of proneural proteins to keep progenitor cells undifferentiated (Bylund et al., 2003). Furthermore, inhibition of Sox2 in VZ progenitors of chick spinal cord leads to cell cycle exit and onset neuronal differentiation (Graham et al., 2003). One of the mechanisms that regulates the balance between self-renewal and differentiation of neural progenitor cell depends on the opposite actions of SoxB1 and related SoxB2 and SoxC family proteins. SoxB2 family members Sox21 and Sox14 together with proneural proteins suppress the activity of SoxB1 and stimulate post-mitotic cell cycle exit (Bergsland et al., 2011;Sandberg et al., 2005;Uchikawa et al., 1999).

1.5.4 The extracellular control of neural progenitor cell proliferation

The extrinsic factors in the environment of progenitor cells are also crucially important for the maintenance of their multipotency. This has been studied both *in vitro* and *in vivo*. In primary rodent telencephalon cultures, Fgf2 promotes the undifferentiated state of progenitors through enhancement of Egf effects (Lillien and Raphael, 2000;Sun et al., 2011). The co-operative mitogenic actions of Fgf2 and Egf are nowadays widely used to

expand neural stem cell numbers in cultures. Fgf2 promotes proliferation through regulation of cell cycle machinery. The upregulation of CyclinD2 shortens G1 phase of the cell cycle and enhances the cyclative mode of progenitors. Simultaneuosly, Fgf2 inhibits Cdk inhibitor p27 expression and subsequently the generation of postmitotic neurons (Lukaszewicz et al., 2002)(Maric et al., 2007).

Conditional inactivation of Fgf receptors (*Fgfr1 and Fgfr2*) in the midbrain leads to premature differentiation of proliferative progenitors causing depletion of proliferative progenitors and ultimately specific midbrain neuronal subtypes (Saarimaki-Vire et al., 2007). The mesodiencephalic dopaminergic neurons (DA) were abolished at perinatal stage in the conditional Fgf receptor mutants (*En1^{Cre};Fgfr1^{cko};Fgfr2^{cko})*. Recently, it was shown that during embryonical development, there appears to be two distinct mesodiencephalic neuronal populations which have differential requirement for Fgf signalling and they utilize diverse genetic programs for differentiation. The midbrain DA precursors are Fgf dependent but, in contrast, the diencephalic DA precursors do not require Fgfs. In the *En1^{Cre};Fgfr1^{cko};Fgfr2^{cko}* embryos the Fgf defiencient midbrain DA precursors adopt diencephalic fate and fail to activate genes important for full maturation of midbrain DA neurons(Lahti et al., 2012). Furthermore, Fgfs were shown to mediate the maintenance of proliferative progenitors by promoting symmetrical proliferative divisions directly from basal side of VZ. Interestingly, in contrast to studies done in vitro, the removal of Fgf signalling in Fgf-receptor mutants did not affect apico-basal cell polarity, cell division plane or cell cycle length. Instead, Fgf signalling seems to be required for the expression of *Hes1* and subsequent repression of neurogenic genes (Lahti et al., 2011). In addition to extrinsic factors in the progenitor cell environment, the contact- dependent signals from differentiating neurons are important for the maintenance of the proliferative progenitor population.

1.6 Coordination of neuronal fate: regulation of neurogenesis

In order to terminally differentiate, a neuronal progenitor cell has to leave the cell cycle and VZ. The ability of progenitors to exit the cell cycle and begin to differentiate is crucial for correct balance and constitution of different neuronal subtypes (Ohnuma et al., 2001). Interplay of different cell cycle regulator complexes coordinates cell cycle progression and determination. In the cortex, Cdk inhibitor p27 has pivotal role in the regulation of neurogenesis as it controls cell cycle exit. Inactivation of *p27* in mice delays the cell cycle exit. A robust increase in the amount of late-born neurons in the cortex of *p27* null mice correlates with reduction in the production of neurons in midcorticogenesis (Goto et al., 2004). In addition to cell cycle exit regulation, p27 has been demonstrated to control differentiation and migration of newborn cortical neurons by stabilizing proneural Ngn2 and inhibition of small GTPase RhoA signalling, respectively (Nguyen et al., 2006).

The most important promoters of neuronal differentiation are proneural proteins originally found essential for Drosophila neurogenesis. Proneural proteins are transcription factors and are thought to act in concert with other cell cycle regulators to promote neurogenesis (Bertrand et al., 2002;Farah et al., 2000). Proneural proteins are character-

ized by sharing motifs of basic helix-loop-helix (bHLH) structure in their DNA binding domain. Based on their dimerization partners, DNA binding motifs and tissue distribution, they can be broadly classified in to two classes, Class A and Class B. Class A, which are also called Class I proteins, are comprised of E-proteins which are generally thought to form heterodimers with Class B proteins. Based on the protein structure and function, Class B proteins can be classified into subclasses, Class II-VII. Class II includes the well characterized proneural proteins Ascl1, Math1, 5, Ngn1-3 and NeuroD which have been shown to be crucial for neuronal development. The positive regulation of neurogenesis and activity of Class II proneural proteins depends on their capability to form heterodimers with E-proteins, belonging to Class A. Heterodimers of bHLH-E-proteins bind to their target sequence containing CANNTG, the E-box region in DNA and predominantly activate the downstream genes important for neuronal differentiation (Bertrand et al., 2002;Massari and Murre, 2000). Several loss and gain of function studies both in invertebrates and vertebrates have shown that proneural proteins are the master regulators of neurogenesis by coordinating several phases of differentiation process in neural progenitor cells (Guillemot, 2007). They promote mammalian pluripotent cells to adopt neurogenic fate, regulate the balance between proliferating and differentiating progenitor cells, promote cell cycle withdrawal and migration of neurons and finally neuronal subtype specification (Bertrand et al., 2002;Castro and Guillemot, 2011;Farah et al., 2000;Fode et al., 2000;Ge et al., 2006;Guillemot, 2007;Nieto et al., 2001;Pacary et al., 2011;Parras et al., 2002).

1.6.1 Notch signalling: Regulation the balance between proliferation and differentiation of neural progenitors

The timing and regulation of proneural gene expression is crucial for normal development of the nervous system. If proliferation and differentiation of progenitors is not balanced, the total number of functional neurons and their diversity are reduced (Hatakeyama et al., 2004;Shimojo et al., 2011). The unique way of ensuring and maintaining a proper progenitor pool during neuronal development is achieved by repressive regulation of proneural genes by Notch signalling. The cellular heterogeneity created by Notch pathway is result of cell-cell interactions between adjacent cells. Progenitor cells that express proneural genes *Ascl*1 or *Ngn2* induce Notch ligand Delta-like1 or Jagged on their cell surface. Delta1 binding to Notch receptor expressed in neighbouring cell triggers the release of intracellular domain of Notch (NICD) which travels to nucleus and forms complex with DNA binding protein Rbpj to induce the expression of bHLH transcriptional repressor *Hes* genes (Kageyama et al., 2008;Shimojo et al., 2011). Hes1, 3 and 5 are thus expressed in the Notch signal receiving proliferative neural progenitor cells. Negative regulation of neurogenesis is achieved by an antagonist effect between *Hes* and proneural genes. Hes1 can actively repress *Ascl1* by binding to its promoter region or directly inhibit Ascl1-E-protein interactions (Chen et al., 1997;Kageyama et al., 2005). Inactivation of *Hes1*, *Hes3* and *Hes5* in mice consistently leads to premature neuronal differentiation, activation of proneural genes and severe defects in several brain structures (Hatakeyama et al., 2004). The Notch pathway is not solely responsible for

regulation of Hes expression. In very early stages of development Hes1 and Hes3 are broadly expressed in the neuroepithelial cells and regional patterning molecules such as Fgf, Shh, Wnt and BMPs are positively regulating Hes expression Notch independently (Kageyama et al., 2009)(Lahti et al., 2011).

In zebrafish retinal progenitors, the movement of progenitor nuclei between the apical and basal side during inter nuclear migration has been suggested to be important for activation of Notch signalling and subsequent correct balance of progenitor proliferation versus differentiation. In the zebrafish retina, Notch is expressed in gradient manner and Notch mRNA expression increases towards the apical surface. In contrast, Delta1 expression is more pronounced in the basal side. The movement of progenitor nuclei between apical and basal sides expose the progenitors to either Notch or Delta depending on the position of the nuclei. In *mok*-mutant retina, the mutation in microtubule-motor-associated- protein, Dynactin 1 disturbes the normal INM kinetics of retinal progenitor nuclei which was suggested to affect the balance between proliferative and neurogenic divisions. As a result of altered INM, the *mok*-mutant retinal progenitor nuclei were less exposed to Notch and this was proposed to lead to premature differentiation of *mok*-mutant retinal progenitors into early-born retinal neuronal subtypes (Del Bene et al., 2008).

Another regulatory mechanism for *Hes* expression involves the autoregulatory capability of Hes1 to bind its own promoter and thereby repress its own transcription. High levels of Hes1 lead to degradation of short-lived Hes mRNA and protein and release Hes regulated negative feedback which enables another round for *Hes* activation. In neural progenitors the oscillatory expression of *Hes1* drives *Delta1* and *Ngn2* oscillations and these complementary oscillations in the expression of Hes and Delta activate Notch signalling in neighbouring cells thereby maintaining a neural progenitor cell pool (Shimojo et al., 2008)(Shimojo et al., 2011) (Figure 3). Hes1 oscillations might act as a cellular clock establishing ability of progenitor cell to either activate or repress downstream target genes required for proliferation or differentiation. Ultimately repression of Notch signalling abolishes Hes1 and establishes sustained activation of proneural proteins and neuronal differentiation (Shimojo et al., 2008)(Kageyama et al., 2009).

1.6.2 Neuronal Subtype specification by proneural proteins

Proneural genes, *Ngns (Ngn1-3*) and *Ascl1*, have important functions also in neuronal subtype specification. Different proneural genes are generally expressed in complementary fashion in the progenitor cells giving rise to specific type of a neuron. There are several different neuronal precursor subtypes, including GABAergic precursors, which require Ascl1 for differentiation. Ascl1 is required for GABAergic neuron development in forebrain, midbrain and dorsal spinal cord (Fode et al., 2000;Kala et al., 2009;Nakatani et al., 2007). On the contrary, complementary expression of Ngns specifies glutamatergic fate in dorsal forebrain and motor neurons in ventral spinal cord (Fode et al., 2000;Gowan et al., 2001;Lee and Pfaff, 2003;Parras et al., 2002).

Proneural genes do not work alone. Instead, during early phases of neurogenesis, they specify different neuronal subtypes in the CNS and PNS together with different

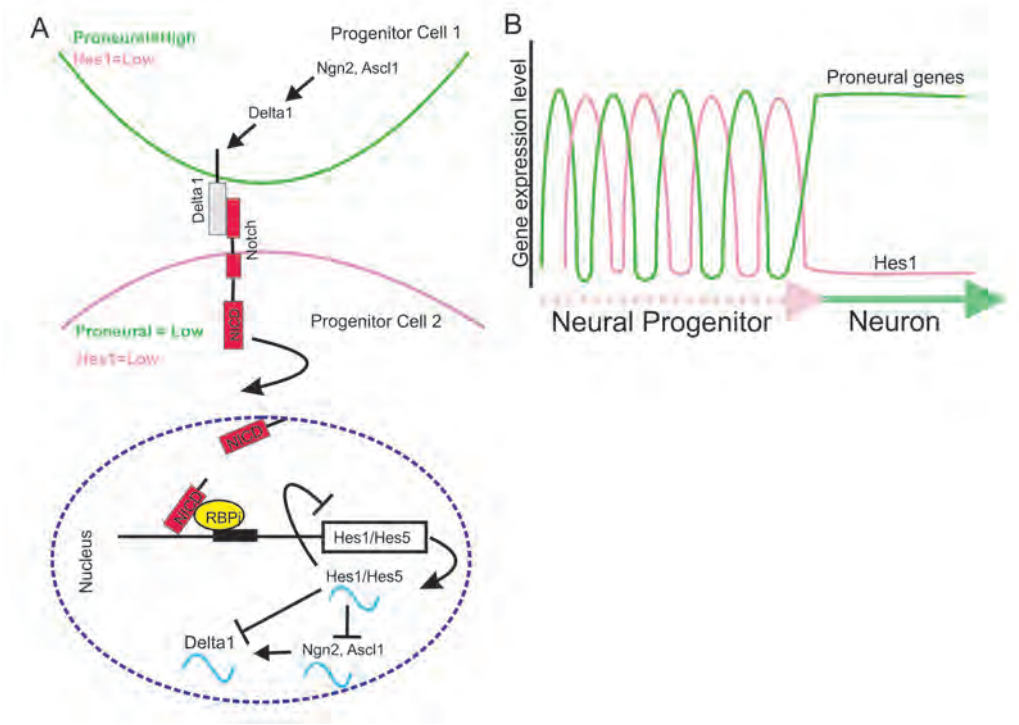


Figure 3. Schematic representation of Notch signalling pathway and dynamic expression levels of *Hes* and proneural genes during neurogenesis. A. The Notch signalling pathway. *Hes* genes are expressed in the proliferative neural progenitor cells and the negative regulation of neurogenesis is achieved by antagonist effect of *Hes* to proneural genes. Proneural genes *Ngn2* and *Ascl1* promote neurogenic differentiation by activating the expression of Notch ligand Delta1 in Progenitor Cell 1 cell surface. Delta1 and Notch receptor interaction leads to release of intracellular domain of Notch (NICD) in Progenitor Cell 2, where NICD is tranferred to nucleus. NICD creates complex with DNA binding protein RBPj and ultimately activates *Hes* genes which repress proneural genes. B. Hes expression oscillates in proliferative progenitors as Hes can autoregulate itself. These oscillations lead to oscillative expression of proneural genes and subsequently oscillation of Delta1. The oscillatory chain reaction maintains the delicate balance between proliferation and differentiation. Downregulation of Hes leads to sustained expression of proneural genes and promotion of neuronal differentiation. Schematic picture is based on (Kageyama et al., 2009)

cell specific transcription factors. In the basal forebrain, Ascl1 together with Dlx1/2 are required for GABAergic subtype specification (Fode et al., 2000)(Bertrand et al., 2002). In the hindbrain and peripheral nervous system Ascl1 specifies noradrenergic phenotype through Phox2a/b transcription factors (Pattyn et al., 2000). In addition Ascl1 is required for serotonergic neuron formation in the hindbrain together with Gata3, Lmx1b and Pet1 (Bertrand et al., 2002;Pattyn et al., 2004). In the midbrain bHLH transcriptional repressor Helt is required together with Ascl1 for GABAergic neuronal subtype formation (Guimera et al., 2006). Neuronal subtype specification is complex process where spatial

and temporal regulation of proneural genes and different co-transcription factors create combinatory effects to induce specific neuronal subtypes in specific regions of developing nervous system (Guillemot, 2007).

1.6.3 Neuronal subtype specification by terminal selector and terminal differentiation genes

As neurogenesis proceeds proliferating progenitors leave the cell cycle and start to express genes that define the morphology and ultimate function of terminally differentiated neuron. Mature neuron, to name a few, has correct morphology and composition of ion channels, functional neurotransmitter synthesizing and transporting proteins and cell surface receptors. Neuronal characteristics are defined by the expression of neuron specific genes, terminal differentiation genes. These genes are activated upon cell cycle exit and are expressed throughout the life of a neuron (Hobert, 2011). Expression of terminal differentiation genes is initiated by terminal selector genes that are transcription factors activated in postmitotic neurons. Inactivation of terminal selector gene leads to downregulation of terminal differentiation genes and ultimately either loss of neuronal identity or transformation of a neuron to another phenotype (Hobert, 2008;Hobert et al., 2010). The terminal selector gene concept has been best established in C. Elegans (Hobert, 2005;Hobert, 2008).

In vertabrates, *Pet1* is terminal selector gene shown to be crucial for defining the serotonergic neuron identity in the CNS and inactivation of *Pet1* leads to severe differentiation defects of postmitotic serotonergic neurons (Liu et al., 2010). Homeobox transcription factor Pitx3 and orphan nuclear receptor Nurr1/Nr4a2 have been suggested to act as terminal selectors for extensively studied midbrain dopaminergic neurons. They are both required for full activation of target genes important for dopaminergic neuron functions (Jacobs et al., 2009). GABAergic neuronal selector genes have been identified in the cerebellum and dorsal spinal cord, where Ptf1a specifies GABAergic fate (Glasgow et al., 2005;Hoshino et al., 2005). In the midbrain, zinc-finger TF Gata2 controls GABAergic neuron formation and in the absence of *Gata2* GABAergic neurons adopt excitatory glutamatergic fate (Kala et al., 2009). The enormous repertoire of different neuronal subtypes in the CNS is ultimately achieved by sequential, combinatory and region specific effect created by proneural, terminal selector and differentiation genes.

1.7 GABAergic neurogenesis

1.7.1 GABAergic neurogenesis in the telencephalon

GABAergic neuron development has been best characterized in the telencephalon. Forebrain is the most anterior structure derived from procencephalon and gives rise to the diencephalon and telencephalon. Telencephalon can be further subdivided into pallium giving rise to cortex and hippocampus and and subpallium that will constitute the more ventral structures, including basal ganglia. GABAergic neurons can be found in subpal-

lium-derived basal ganglia, where they participate in the regulation of motor coordination, but also in pallium-derived structures, cortex and hippocampus, where GABAergic interneurons adjust the activity of excitatory pyramidal neurons.

In the forebrain, as in all regions of the CNS, progenitor differentiation and subsequent neuronal subtype specification from progenitor cells is dependent on the correct A-P and D-V patterning of early neural tube. Early patterning events are regulated by combinatory interactions of common spatial patterning molecules. The regional patterning is followed by complementary expression of different proneural proteins and subtype specification transcription factors, which regulate brain development region specifically. The expression of distinct transcription factors in neural progenitor cells creates progenitor domains that give rise to different neuronal subtypes. Forebrain can be divided into distinct progenitor domains according the expression of HD transcription factors Nkx2.1, Nkx6.2, Pax6, Gsh1/2 and Dlx1/2 together with proneural proteins Ngn2 and Ascl1.

GABAergic neurons are born in the ventral subpallium which can be divided into medial (MGE), lateral (LGE), caudal (CGE) and septal (SGE) ganglionic eminence (Jovanovic and Thomson, 2011). In the ganglionic eminences, the differences in gene expression of different TFs give rise to distinct subtype populations of cortical GABAergic neurons, which can be characterized by expression of co-neurotransmitters and calcium binding proteins such as neuropeptide Y, calbindin, parvalbumin and calretinin (Jovanovic and Thomson, 2011). Initial GABAergic fate commitment is dependent on the activity of homeodomain transcription factors Dlx1/2 and proneural protein Ascl1. Inactivation *Dlx1* and *Dlx2* function in the mice have revealed their redundant roles in regulating forebrain GABAergic neuron fate and migration. *Dlx1/2* mutant mice have lost GABAergic interneurons in neocortex, hippocampus, olfactory bulb and striatum (Anderson et al., 1997;Bulfone et al., 1998;Cobos et al., 2005;Pleasure et al., 2000). Ascl1 is expressed in the progenitor domains of MGE and LGE and inactivation of *Ascl1* alone leads to loss of specific subpopulations of GABAergic interneuron in the cortex and basal ganglia. Despite the loss of GABAergic interneurons produced in MGE, the LGE progenitors were unaffected. This demonstrates that Ascl1 is required for GABAergic neuron formation, even though the requirement is region restricted (Casarosa et al., 1999). The functions of proneural proteins are broad (see 1.6.2) and Ascl1 likely regulates region specifically different aspects of progenitor cell differentiation and specification together with other co-factors.

After cell cycle exit, the immature forebrain GABAergic interneurons migrate tangentially to their final positions in the cortex and basal ganglia where GABAergic neurons populate the ventral telencephalon, including striatum (Campbell, 2003;Marin and Rubenstein, 2001). The tangential migration is a complex process and is regulated by multiple different transcription factors, cell surface receptors and chemical cues. Migrating cortical GABAergic neurons follow specific tangential routes to their final positions and travel across regional forebrain boundaries (Marin and Rubenstein, 2003). The early born interneurons follow ventral route that travels along marginal and intermediate zone underlying cortex. Late born interneurons migrate more dorsally following route that travels through subventricular zone (Jovanovic and Thomson, 2011). The molecular interplay that regulates tangential migration can be divided into three main categories:

factors stimulating movement (motogenic), structural factors and directional cues from the environment of interneurons (Marin and Rubenstein, 2003).

Several motogenic factors stimulate the movement of immature neurons and among the most definite ones are different growth factors and neutrophins. In vitro studies with brain slice cultures of cortex and ganglionic eminences demonstrated that Brain-derived neutrophine factor (BDNF) and neutrophin-4 (NT-4) regulate interneuron motility from MGE to cortex. BDNF and NT-4 are ligands for TrkB receptors expressed on the cell surface of migrating MGE interneurons. Subsequently, migration is stimulated by TrkB receptor-mediated downstream signalling cascades in the migrating interneurons (Polleux et al., 2002). Another growth factor, Glial cell derived neutrophine factor (GDNF), promote tangential migration of MGE derived GABAergic interneurons. GDNF-stimulated migration is mediated trough GFR1a receptor signalling and in addition to migration, GDNF contributes to morphological differentiation of cortical GABAergic neurons (Pozas and Ibanez, 2005). Hepatocyte growth factor/scatter factor (HGF/SF) regulates interneuron migration and cortical interneuron subtype specification via urokinase plasminogen activator (uPAR) receptor (Powell et al., 2001;Powell et al., 2003).

Compared to rostral migratory stream and interneuron migration into olfactory bulb, fairly little is known of the structural substratum utilized by tangentially migrating interneurons. It has been suggested that outward passing nerve fibers from the cortex serve as a platform for tangentially migrating interneurons. Futhermore, the routes of tangentially migrating interneurons are concentrated to the axon sparse areas and therefore the substrates that support tangential migration remains to be solved (Marin and Rubenstein, 2003;Nakajima, 2007).

Chemical cues can act as a repellent or attractant to instruct the interneuron cell migration along different routes. Semaphorins (Sema 3A and Sema 3F) are expressed in the LGE and mediate repulsive actions to interneurons destined to cortex thus inhibiting their entry to basal ganglia. The repulsive mechanism for Sema 3A/F mediated segregation of cortex versus striatum interneurons is based on the expression of neuropilins. Interneurons migrating to cortex express neuropilins in their cell surface which make them responsive for Sema 3A/F repulsion, on the contrary, interneurons destined to striatum, lack neuropilins from their cell surface thus enabling them to enter striatum (Marin and Rubenstein, 2001). Another example of factors guiding interneuron migration are Slit-Robo proteins. Slit proteins together with their Robo receptors are expressed in the VZ and SVZ of ganglionic eminence and Robo1 and Robo2 in the migrating cortical interneurons. It has been suggested that Slit acts as chemical repellent in the VZ and SVZ guiding migrating interneurons away from the ventricular surface (Zhu et al., 1999). The exact mechanism of Slit-Robo mediated migration guidance is still under investigation, although interneurons in the cortex and striatum of Slit-Robo-mutant mice show altered morphology and location (Andrews et al., 2008;Andrews et al., 2007).

The attractants expressed in the cortical areas are important for directing migrating neurons to their final destinations. Stromal-derived factor 1 (SDF-1) expressed in MZ/SVZ act as chemoattractant to migrating interneurons which express SDF-1 receptor CXCR4. SDF-1/CXCR4 signalling stimulates tangential migration within the cortex and regulates interneuron dispersal into their correct layers. (Lopez-Bendito et al., 2008;Tiveron et al., 2006).

Finally, neurotransmitters participate to the regulatory network of molecules that guide interneurons during their long journey to and within the cortex. In several studies neurotransmitter GABA has been shown to regulate several steps in the neuron migration. GABA modulates cortical neuron migration concentration dependently (Behar et al., 1996). GABA is found in the migratory paths from subpallium to cortex and promotes cortical entry of tangentially migrating MGE derived interneurons which express GABA-A receptor on their cell surface (Cuzon et al., 2006). Once interneurons reach cortex they invade different layers of cortical plate. GABA-A receptor mediated depolarization in migrating interneurons has been shown to induce interneuron motility. As discussed in 1.3, GABA can mediate depolarization in developing neurons. In motile interneurons, the expression of KCC2-transporter is low. There GABA depolarizations lead to accumulation of intracellular Ca^{2+} by the activation of voltage sensitive calsium channel (VSCC) (Bortone and Polleux, 2009). Subsequent upregulation of KCC2-transporter in interneurons that reach cortex reduces migration by hyperpolarizing cells trough GABA-A receptor (Bortone and Polleux, 2009). Therefore GABA can have distinct paracrine effects on migrating interneurons depending on their state of maturation. GABA induces motility by depolarizations in immature migrating interneurons that express low levels of KCC2, while in more mature interneurons expressing higher levels of KCC2; GABA can terminate migration as the cells reach their correct positions in the cortex.

Another neurotransmitter, dopamine and its receptors dopamine receptor D1 and D2 are abundantly expressed in the striatum and LGE of basal forebrain (Ohtani et al., 2003) . The knock-out (KO) mice of dopamine receptor D1 mice have significantly less GABAergic interneurons in their cerebral cortex. It was shown that dopamine receptor D1 activation promotes GABAergic interneuron migration from basal forebrain to cerebral wall (Crandall et al., 2007). In contrast, dopamine receptor D2 decreases GABAergic neuron migration and these opposing actions of dopamine receptor D1 and D2 activation have also shown to modulate cell cycle progression in the embryonic telencephalon (Ohtani et al., 2003) . The delicate balance of dopamine receptor D1 and D2 expression and activation is crucial for correct GABAergic neuron migration and normal brain development (Crandall et al., 2007;Ohtani et al., 2003).

1.7.2 Gabaergic neurogenesis in the developing diencephalon

Diencephalon is posterior part of the forebrain that contains important structures such as the thalamus, hypothalamus and the posterior portion of the pituitary gland. Diencephalon contains several sensory nuclei that serve as relay station to receive sensory information from periphery and transmit projections to corresponding cortical areas. GABAergic neurons are found in several regions of the mature and developing diencephalon. Ventral lateral geniculate nucleus (vLGN) and intergeniculate leaflet (IGL) are important diencephalic GABAergic nuclei that receive inputs from retina, superior colliculus and many other brain areas. They act as visuomotor integration centers and are thought have a role in circadian rhythm regulation together with suprachiasmatic nucleus in the hypothalamus (Harrington, 1997).

During embryonic development diencephalon is subdivided into different domains, prosomeres; P1/pretectum, P2/thalamus, P3/prethalamus and organizing center Zli, which is formed in the boundary between P2 and P3. GABAergic marker genes are expressed at early embryonic development in P1, rostral part of P2 and in P3 domains which later give rise to GABAergic neurons (Hashimoto-Torii et al., 2003;Kataoka and Shimogori, 2008;Kitamura et al., 1997). Domain specific expressions of different transcription factors together with molecules secreted from Zli regulate the early differentiation of proliferative progenitors in the diencephalon. They induce the expression of proneural genes *Ngn1/2* and *Ascl1* regulating progenitor cell cycle exit and neuronal subtype specification. During embryonic development the excitatory glutamatergic neurons and inhibitory GABAeric neurons are differentiated in complementary regions of P2.

In zebrafish bHLH transcription factor Her6 is initially expressed in the entire presumptive thalamus region and later in development the expression is restricted to the Ascl1 positive neuronal progenitors of rostral P2 (p-Th-R) and P3. Her6 was shown to acts as repressor of *Neurogenins*, which in cortex are required for glutamatergic neuron development. Her6 mediated repression of *Ngns* subsequently promote *Ascl1* expression and GABAergic neuron differentiation (Scholpp et al., 2009). Ascl1 is region specifically required for GABAergic neuron differentiation in the forebrain and it is expressed in the P1, rostral part of P2 (p-Th-R) and P3, domains giving rise to GABAergic neurons in diencephalon (Kataoka and Shimogori, 2008;Vue et al., 2007)(Fode et al., 2000). Inactivation of *Ascl1* was reported to lead to loss of GABAergic neurons in brain regions posterior to Zli (Miyoshi et al., 2004). Otx2 promotes glutamatergic fate specification in thalamus. Ectopic upregulation of Ascl1 and subsequent glutamatergic to GABAergic neuronal identity switch in Otx2 deficient progenitors further supports a role for Ascl1 in the differentiation of GABAergic neurons in diencephalon (Puelles et al., 2006).

1.7.3 GABAergic neurogenesis in the developing midbrain

In the mature midbrain GABAergic neurons can be divided into functional and anatomical subpopulations. In the ventral most midbrain there are important GABAergic neurons intermingled with dopaminergic neurons (DA) in the Substantia Nigra pars reticulata (SNpr) and Ventral Tegmental Area (VTA). Dopamine is known as a neurotransmitter that regulates motivational responses and motor coordination of an individual. Substantia Nigra (SN) and VTA DA neurons innervate and release dopamine into cerebral cortex, striatum and limbic forebrain (Bjorklund and Dunnett, 2007). Depending on the type of stimuli a person experiences, whether rewarding or non-rewarding, the midbrain dopaminergic neurons have differential response and mode of neurotransmitter transmission (Bromberg-Martin et al., 2010)(Cohen et al., 2012). Although the dopamine system has gained a lot of attention in the research field, SN-VTA neurons are not solely dopaminergic as 90% of SN and only 60% of VTA neurons are dopaminergic (Fields et al., 2007). In the VTA and surrounding areas, there are abundant GABAergic neuron population, which convey inhibitory input to SN-VTA dopaminergic neurons and regulate their activity. The mechanism behind addictive drugs is based on inhibition of these GABAergic neurons thereby increasing dopamine release in the brain. Important

pathway regulating behavioural responses to unpleasant events is mediated by lateral habenula (LHb). Several subcortical and hypothalamic nuclei send inputs to LHb. The activation of LHb has been shown to inhibit SN-VTA DA neuron activity (Matsumoto and Hikosaka, 2007). This inhibition is mediated by rostromedial tegmental nucleus (RMTg) which is located in the mesopontine area of the midbrain. RMTg is mainly composed of GABAergic neurons which receive afferent input from several subcortical nuclei such as amygdala and vPAG (Jhou et al., 2009b). RMTg-mediated GABAergic input to SN-VTA DA is activated in aversive circumstances. GABA-mediated inhibition is important signal to decrease dopamine cell firing which subsequently is suggested to influence decision making of an individual (Jhou et al., 2009b)(Jhou et al., 2009a)(Hong et al., 2011)(Cohen et al., 2012).

Other important midbrain GABAergic nuclei include medially located mesencephalic reticular formation (mRF) the function of which is not fully characterized and periaqueductal gray (PAG) which modulates nociceptive stimuli by regulating the activity of neurons in the pain mediation pathway. The most dorsal GABAergic neurons locate in the superior and inferior colliculus (SC, IC) and are involved in the regulation sensory information.

Midbrain GABAergic neurons consist of several anatomical and functional subpopulations, which mediate important brain functions. Despite the functional importance their molecular and developmental regulatory mechanisms are still rather poorly understood. During embryonic development midbrain can be divided dorsoventrally into progenitor domains (m1-m7) based on the region specific expression of different transcription factors (Kala et al., 2009;Nakatani et al., 2007). In the production of GABAergic neurons during embryonic development, there is temporal difference between ventral and dorsal midbrain. The most ventro-lateral domains m5-m3 produces GABAergic neurons at early stages of embryonic development and dorsal domains m2-m1 give rise to GABAergic neurons in later developmental stages (Nakatani et al., 2007).

Recently, the molecular interplay guiding GABAergic neurogenesis in the midbrain has begun to be understood in more detail. Proneural protein Ascl1 together with bHLH transcription factor Helt and zinc-finger transcription factor Gata2 regulate different stages of the GABAergic neurogenesis in the midbrain. Helt and Ascl1 are co-expressed in the proliferating progenitor cells that give rise to GABAergic neurons in the midbrain and caudal diencephalon. Misexpression of Helt induces prematurely ectopic GABAergic neurons in Ascl1 expressing progenitors in the dorsal midbrain that normally differentiate later in the development (Miyoshi et al., 2004). Furthermore, inactivation of *Helt* leads to loss of Gad1 expression and GABAergic neurons specifically in the superior and inferior colliculus. As a result of reduced GABAergic activity the *Helt* mutant mice showed seizures, growth reduction and increased postnatal death (Guimera et al., 2006). The mechanism of Helt function has been revealed by gain and loss of function studies. Helt represses *Ngn1/2* expression and subsequently suppresses glutamatergic phenotype, as in the *Helt* mutant midbrain the regions normally giving rise to GABAergic neurons are expressing glutamatergic markers. Therefore Helt can determinine GABAergic over glutamatergic neurotransmitter phenotype in the proliferative progenitors (Nakatani et al., 2007).

There is limited amount of studies on the requirement of Ascl1 in the midbrain GABAergic neurogenesis. Previously, it was suggested that Ascl1 is absolutely required for GABAergic neuron differentiation in the midbrain as no Gad1 expressing GABAergic neurons could be found from *Ascl1* deficient mice (Miyoshi et al., 2004). However, in this study the authors characterized only early embryological time point of GABAergic neurogenesis.

Zinc-finger (ZF) transcription factor Gata2 expression is initiated in the midbrain GABAergic neuronal precursors as they exit the cell cycle. Conditional inactivation of *Gata2* in the mouse midbrain does not affect cell cycle exit but transforms GABAergic precursors into glutamatergic fate. In these *Gata2* mutants the post-mitotic activation of genes required for GABAergic differentiation failed completely in the midbrain. Instead, genes typical for glutamatergic differentiation were activated. Interestingly, the GABAergic neurons in the VTA-SNpr were not affected (Kala et al., 2009).

After the final subtype specification, the immature GABAergic neurons must migrate to their final destinations and constitute specific GABAergic nuclei. Based on the sparse studies of the mechanisms regulating midbrain GABAergic neuronal migration it seems, to some extent, be different compared to the forebrain. In the midbrain the dorsal neuroepithelium (m1-m2) also produces GABAergic neurons. Midbrain GABAergic neurons predominantly migrate radially although tangential migration might operate after the radial migration has been completed. In mouse where GABAergic cells were visualized with knock-in of GFP into Gad67 promoter, the neurons migrated predominantly radially between ventricular and pial surfaces in the superior colliculus (Tsunekawa et al., 2005).

1.7.4 GABAergic neurogenesis in the anterior hindbrain and cerebellum

During embryogenesis hindbrain is subdivided anteroposteriorly into seven segments called rhombomeres (r1-r7). These segments are separated by the segment specific expression of *Hox* genes. The most anterior segment, rhombomere1, is delineated posteriorly by the expression of *HoxA2* and anteriorly by patterning signals from MHB (Irving and Mason, 2000;Joyner et al., 2000). The layered structure of cerebellum is constructed from dorsal rhombomere 1, which gives rise to outer molecular layer (ML), Purkinje cell layer (PC), granule cell layer (EGL) and deep cerebellar nuclei (DCN) (Joyner and Zervas, 2006). The ventral rhombomere 1 together with other rhombomeres compose specific nuclei such as pontine, vestibular and cuneate nuclei. During embryonic and postnatal development cerebellar neurogenesis follows spatial and temporal regulation. The two principal neuronal subtypes are derived from the dorsal VZ and rhombic lip (RL) which give rise to GABAergic and glutamatergic neurons, respectively (Carletti and Rossi, 2008). Glutamatergic granule cells and deep nuclear projection neurons are derived from the dorsal RL. Differentiation of glutamatergic neurons is regulated by transcription factor Math1 (Carletti and Rossi, 2008).

The VZ derived GABAergic neurons follow strict sequential order in their differentiation as the deep nuclear neurons and Purkinje cells are born first following the embryonic and postnatal birth of local interneurons such as stellate and basket cells (Carletti

and Rossi, 2008). The master regulator of GABAergic neuronal subtype specification in developing cerebellum is bHLH transcription factor Ptf1a (Hoshino et al., 2005). Ptf1a is expressed on the progenitor cells of the cerebellar VZ and its inactivation leads to inhibition of GABAergic neuronal subtype formation from cerebellar VZ. With the exception of inferior olivary and pontine nucleus which send projections to cerebellumas well as dorsal spinal cord, Ptf1a was shown to be specifically required for GABAergic subtype selection of the cerebellum as in other regions such as inferior colliculus the generation of GABAergic neurons was unaffected (Hoshino et al., 2005). Very little is known about GABAergic neurogenesis in the ventral rhombomere1. Ventral r1 contains several nuclei important for respitatory control and motor coordination (Gray, 2008;Grossmann et al., 2010). Neurons with different neurotransmitter phenotypes such as serotonergic, GABAergic and cholinergic localize in the ventral r1. Recently, Waite et. al delineated a map of ventral r1 based on the expression of several distinct transcription factors and identified of a paired-like homeodomain transcription factor Pitx2 expressing GABAergic domain in the ventral r1 (Waite et al., 2012).

1.7.5 GABAergic neurogenesis in the developing spinal cord

The spinal cord is the most extensively studied part of developing neural tube where the signalling pathways instructing the region specific regulation of neuronal differentiation are understood in great detail. The rostrocaudal and dorsoventral distribution of spinal neuronal differentiation signals produces specific neuronal subtypes at correct positions along developing spinal cord. GABAergic interneurons are localized into both dorsal and ventral horn of the spinal cord. In the dorsal regions GABAergic neurons are involved in the processing and relaying the peripheral sensory signals and ventrally regulating the activity and output of motor neurons. The dorsal spinal cord progenitor domains that give rise to both glutamatergic and GABAergic neurons are divided into subtypes based on the requirement for specification signals. Differentiation of dorsal interneurons belonging to Class A (dI1-3) is dependent on the roof plate secreted molecules such as BMPs and Wnts. On the contrary, interneurons belonging to Class B (dI4-6 dILa and dILb) differentiate roof plate independently (Lee and Jessell, 1999)(Pfaff and Macfarlan, 2009).

Interneurons dILa and dILb are born later compared to early born dI1-6 neurons. These late born neurons belong to both GABAergic (dILa) and glutamatergic (dILb) subtypes and they express Pax2/Lhx1/5 and Tlx1/3 and Lmx1b, respectively (Cheng et al., 2004;Gross et al., 2002). Tlx1/3 has been shown to regulate glutamatergic subtype specification in the dorsal horn (Cheng et al., 2004). Late born GABAergic neurons (dILa) are specified by Ascl1 which regulates *Ptf1a* expression and Notch signalling and induces the differentiation of dILa interneurons from single pool of progenitors (Mizuguchi et al., 2006). Inactivation of *Ptf1a* leads to loss of dorsal spinal horn GABAergic neurons and accumulation of glutamatergic neurons (Glasgow et al., 2005). The early ventral spinal cord patterning is a result of graded Shh signalling which creates specific progenitor domains and together with domain specific HD TFs control the subsequent subtype specification. Progenitor domain p2 gives rise to both glutamatergic

and GABAergic neurons named V2a and V2b, respectively (Karunaratne et al., 2002). As in the dorsal spinal cord, neuronal subtype specification in the ventral spinal cord depends on mutual repressive interactions of several different transcription factors that define the differentiation of specific neuronal subtypes into correct positions. Winged-helix/forkhead TF Foxn4 is expressed in the p2 progenitors that give rise to both types of V2 neurons (V2a and V2b) (Del Barrio et al., 2007;Li et al., 2005). Initially, Gata2 is needed for production of both types of V2 neurons. Segregation of two distinct neuronal subtypes from common progenitor pool is result of regulatory network of Foxn4, Ascl1, Gata2 and Notch/Delta4 signalling (Del Barrio et al., 2007;Li et al., 2005;Zhou et al., 2000). The immature V2 precursors express Foxn4, Gata2, Ascl1 and Lhx3. Subsequent reciprocal expression of Notch/Delta4 in neighboring cells initiates the cascade that leads to segregation of two different subtypes, precursor that have activated Notch and another cell population with activated Dll4. Precursor cells with activated Notch, Foxn4, Ascl1 and Gata2 differentiate into GABAergic V2b neurons. The complementary cell without activated Notch, adopt V2a fate and trough Delta4 switch on the V2a subtype specification (Del Barrio et al., 2007). The final steps in the V2b GABA subtype specification are regulated by Lmo4, Tal1 and Gata2/3 (Joshi et al., 2009;Peng et al., 2007;Zhou et al., 2000).

1.8 Molecular and Biological functions of transcription factors Ascl1, GATA2/3 and Tal1/2

1.8.1 Molecular biology of Ascl1

The HLH proteins regulate several diverse processes during the development of an individual. They have been identified in the regulation of cell proliferation, differentiation and subtype specification of distinct organs. Their ability to regulate such diverse processes is achieved by the ability to form heterodimers with other proteins and to bind specifically to target sequences in the DNA (Bertrand et al., 2002;Massari and Murre, 2000). The bHLH protein Ascl1 belongs to neurally expressed subset of class II bHLH transcription factors and act as transcriptional activator. Ascl1 forms heterodimers with another bHLH protein E-protein (E12/E47) and binds to CANNTG target sequence in the DNA, called E-box (Johnson et al., 1992;Massari and Murre, 2000). The basic region of bHLH protein binds to the main groove of DNA and makes contacts with target sequence. The HLH domain which consist four α-helixes of heterodimerized proteins is responsible for the dimerisation of HLH proteins (Bertrand et al., 2002).

Although it is widely accepted that proneural proteins regulate multiple steps during neurogenesis, fairly little is known of the exact molecular pathways conveying their functions. Ascl1 function during neurogenesis was clarified very recently (Castro et al., 2011). In this study, authors combined genomic location of Ascl1 binding and expression profiling of *Ascl1* mutant and overexpressing embryos to elucidate Ascl1 transcriptional target genes. As expected, it was revealed that Ascl1 directly regulates wide array of target genes which have distinct molecular and cellular functions during neurogenesis. These target genes have been shown to regulate neuronal differentiation, subtype

specification, signal transduction, cytoskeleton regulation, neurotransmitter biosynthesis and cell cycle control. Surprisingly, the expression profiling and chromatin immunoprecipitation analysis of both cultured neural stem cells and embryonic telencephalic cells revealed that Ascll, in addition to genes driving cell cycle exit, also activates positive cell cycle regulators. Thus, Ascl1 has unique ability to activate transcriptional programs that activate target genes in both cell cycle progression and termination and therefore the entire program of neurogenesis. The mechanism how Ascl1 selects, activates and suppresses such diverse target genes during neurogenesis is still unknown. Presumably there are several cofactors and pathways co-operating and regulating Ascl1 function.

1.8.2 GATA and Tal transcription factors

ZF transcription factor family GATA consist of six family members named GATA1-6. All the members of this protein family contain conserved zinc-finger structure in the DNA binding domain which mediates their binding to the consensus (A/T)GATA(T/G) sequence (Patient and McGhee, 2002). Based on the expression patterns in different tissues, GATA family can be subdivided in to two families. GATA1, 2 and 3 were first described in the hematopoietic system but GATA2 and GATA3 also have important roles in the development of nervous system. GATA4, 5 and 6 are expressed in the mesoderm and endoderm derived structures such as cardiac, gastrointestinal and gonadal tissues (Molkentin, 2000).

GATA family members are highly identical by their amino acid sequence in the zinc-finger domain responsible for DNA binding and they all can identify and bind to the same target consensus sequence. Regardless of their homologous protein structure and ability to bind the same target sequences, GATA factors can context-dependently differentially regulate same target genes with opposing outcomes (Bresnick et al., 2005;Wozniak et al., 2008). This ability is based on the protein structure that in addition to mediating protein-DNA binding, convey protein-protein interactions (Weiss et al., 1997). Several studies have shown that GATA factors interact with different cell-specific cofactors. In the hematopoietic system, pluripotent progenitor cells continuously create more committed precursor cell types of mature blood cells (Cantor and Orkin, 2002). During hematopoietic differentiation the transcriptional activity of GATA factors is based on their ability to physically form multiprotein transcription complexes with several different proteins. The bHLH TF Tal1 (also known as SCL), Lim-only protein Lmo2 and GATA 1, 2 and 3 play pivotal roles in the regulation of embryonic hematopoiesis (Shivdasani et al., 1995;Wadman et al., 1997;Yamada et al., 2000). Like other bHLH proteins, Tal1 forms heterodimers with ubiquitously expressed E-proteins such as E12/E47 and bind to target E-box sequence CANNTG (Hsu et al., 1994). In addition to binding DNA, Tal1 interacts with various other proteins such as Lmo2. Lmo2 functions as a bridging molecule between Tal1 and GATA which mediate the binding of multiprotein transcriptional complex to DNA target sequences. Mice lacking *Tal1*, *Lmo2* or *Gata2* show similar phenotype and die during early embryogenesis due to failure in the hematopoietic development (Cantor and Orkin, 2002;Shivdasani and Orkin, 1996). The similar phenotypic outcomes are result of inability to form Tal1/E47/Lmo2/NLI/

GATA multiprotein transcriptional complex regulating crucial steps in the embryonic hematopoietic development (Wadman et al., 1997).

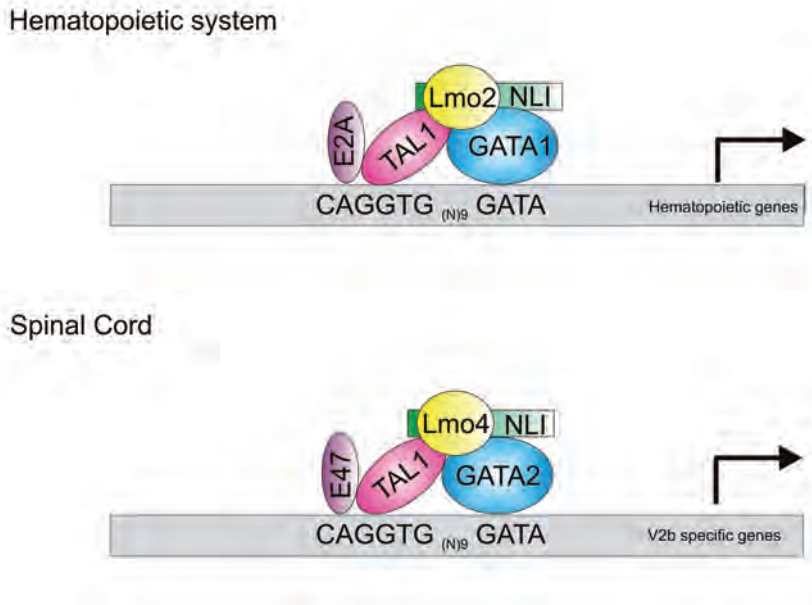


Figure 4. Model representing the transcriptional multiprotein complex of GATA/TAL. Such complexes regulate development hematopoietic system and the specification of V2b GABAergic neurons of the spinal cord.There are potentially several different complexes with distinct protein partner composition acting during different stages of hematopoiesis and other cell type specifications. DNA binding of the complex is mediated by heterodimer of E-protein and TAL1 (E2A-TAL1) binding to E-box region of the DNA and GATA binding to its conserved target sequence (GATA). LMO and NLI proteins act as linker protein by connecting the two DNA binding motifs together. (Model is based on Joshi et al., 2009;Wadman et al., 1997)

In addition to their well documented roles in the hematopoietic development, Gata2, Gata3 and Tal1 are expressed in the CNS and regulate neuronal differentiation and subtype specification (Smith et al., 2002). The expression of *Gata2* begins at E9.5 and precedes that of *Gata3* which starts to be expressed one day later at E10.5. Gata2 positive neural precursors can be found in VZ and IZ. Gata3 expression is restricted to MZ. *Gata3* expression is dependent on the expression of Gata2 and they are both expressed in several regions of developing CNS including olfactory bulbs, pretectum, midbrain and caudal spinal cord (Nardelli et al., 1999)(Kala et al., 2009) *Gata2* acts as a post-mitotic selector gene for GABAergic neurons in the midbrain (Kala et al., 2009) and together with Gata3 regulates cell type specification of V2b interneurons in the spinal cord and differentiation of serotonergic neurons in hindbrain (Craven et al., 2004;Karunaratne et al., 2002).

The bHLH transcription factor Tal1 was initially identified as leukemogenic factor (Brown et al., 1990;Chen et al., 1990) During embryogenesis it is expressed in the intermediate and marginal zone of diencephalon, midbrain and hindbrain (van Eekelen et al., 2003). Conditional inactivation of *Tal1* in neuronal cells has revealed its requirement for neuronal development (Bradley et al., 2006). *Nestin-Cre;Tal1* $^{Flox/Flox}$ mice are growth-retarded, hyperactive, display altered motor coordination and die prematurely. The effect of *Tal1* neuronal ablation is region-specific since the most pronounced loss of normally Tal1 expressing cells is seen in the ventral hindbrain including the interpeduncular nucleus (IP), dorsal tegmental nucleus (DTg), laterodorsal tegmental nucleus (LDTg) and dorsal and ventral tegmental nucleus (DTg, VTg). Detailed cell type specificity and developmental mechanisms were not addressed in this study although, in the *Nestin*$^{-Cre}$*;Tal1* $^{Flox/Flox}$ mice the number of GABAergic neurons was significantly reduced in the ventral hindbrain. The partial reduction of Tal1 expressing cells in the midbrain indicates possible functional compensation by another protein. Indeed, related bHLH TF Tal2 is expressed in diencephalon, midbrain and hindbrain (Mori et al., 1999). Tal2 bHLH region is highly homologous to that of Tal1 and their expression domains partially overlap in the diencephalon and midbrain although Tal2 expression is more pronounced in the proliferative zones of neuroepithelium and the expression precedes that of Tal1 expression. In the hindbrain weak expression of Tal2 is confined in the anterior regions compared to Tal1 expression which is expressed in more posterior regions such as posterior pons and myelencephalon (Mori et al., 1999)(Green and Begley, 1992). Homozygous mutants of *Tal2* do not have defects in haematopoiesis although *Tal2* activation by chromosomal translocation has been associated with subset of T-cell leukemogenesis (Xia et al., 1991)(Bucher et al., 2000). *Tal2*-ablated mice are growth retarded and die prematurely. The anatomical defects include dysgenesis of the midbrain dorsal structures; size reduction of superior colliculi and protrusion of inferior colliculi. The plausible cause of death is hydrocephalus and subsequent compression of vital brain structures (Bucher et al., 2000). In this study the authors did not characterize the cell type specificity behind the observed defects.

The expression patterns and region specific defects in neuronal ablated *Tal1* and homozygous *Tal2* mutants indicate their redundant roles during neuronal development. Tal family members Tal1 and Tal2, Gata2 and Gata3 are expressed in partially overlapping regions of developing diencephalon, midbrain and hindbrain. In the hematopoietic system and spinal cord Gata1/2 forms multiprotein transcriptional complex with Tal1 and regulates the subtype specification of hematopoietic cells and spinal interneurons respectively (Joshi et al., 2009;Wadman et al., 1997; Figure 4). The expression patterns and mutational analysis of both Gata2 and Tal1 in the midbrain and anterior hindbrain suggest that similar transcriptional complex potentially regulate the neuronal differentiation and subtype specification in these brain regions.

2. AIMS OF THE STUDY

In this study we aimed to understand the developmental processes and molecular pathways regulating GABAergic neuron differentiation in the diencephalon, midbrain and anterior hindbrain. We have been especially interested in the development of GABAergic neurons associated with the midbrain dopaminergic VTA-SN area. These GABAergic neurons are essential in the regulation of the activity of dopaminergic pathways which have well known functions in the control of voluntary movements and regulation of behaviour. We aimed to investigate the molecular mechanisms of GABAergic neurogenesis regulated specifically by different transcription factors during diencephalic, midbrain and hindbrain development.

Our specific aims were:

1. To study the involvement of the proneural gene *Ascl1* in the development of the midbrain GABAergic neurons.
2. To study the functions of Gata2 and Ascl1 in GABAergic neurogenesis in the developing diencephalon.
3. To study how the Gata2 protein partners Tal1 and Tal2 are involved in the regulation of GABAergic neurogenesis in the midbrain and anterior hindbrain.
4. To analyze developmental origin of VTA-SNpr GABAergic neurons.

3. MATERIALS AND METHODS

3.1 Methods

Methods and mouse lines used in this study are listed in Table I and II. mRNA in situ hybridisation analyses on paraffin sections were performed using either digoxigenin or ^{35}S- labelled cRNA probes. Antibodies and in situ hybridisation probes can be found from the Methods sections of studies I-IV. To support the validity of stainings a suitable positive and/or negative control were included in the in situ hybridazation and immunohictochemistry procedures.

Table 1. Methods used in this study.

Method	Reference / Source	Study number
ISH on paraffin sections	(Wilkinson and Green, 1990)	I-IV
IHC on paraffin sections	(Kala et al., 2008)	I-IV
combined ISH and IHC	See study	III
BrdU labelling	See studies	I,II,IV
Birth dating by BrdU labelling	See study	IV
Tamoxifen administration	See study	IV
PCR genotyping	Original publications in Table 2	I-IV
Statistical methods	See study	IV
Microscopy and quantification	See studies	I-IV

3.2 Materials

Table 2. Mouse lines used in this study.
The PCR primers and expected PCR products can be found from original publications. For embryonic staging, vaginal plug was counted as embryonic day 0.5 (E 0.5). Inducible Cre, the *Gbx2*^{CreERT2} was induced by single dose of tamoxifen (3µg/30g of body weight) either intraperitoneal injection at E 8.5 or by oral dosage at E9.5. All animal procedures were performed in accordance with the Finnish national guidelines and instructions. The expreriments were approved by the committee of experimental animal research of the University of Helsinki.

Table 2. Mouse lines used in this study.

Mouse line	Reference / Source	Description	Study number
Ascl1^{KO} (Mash1null)	(Guillemot et al., 1993)	Null allele of *Ascl1*	I
Foxg1-Cre	(Hebert and McConnell, 2000)	*Cre-recombinase* under *Foxg1* promoter	II, IV
En1-Cre	(Kimmel et al., 2000)	*Cre-recombinase* under *Engrailed1* promoter	I-IV
Gbx2-Cre^{ERT2}	(Chen et al., 2009)	*Inducible Cre-recombinase* under Gbx2 promoter	IV
Shh-Cre	(Harfe et al., 2004)	*Cre-recombinase* under *Sonic hedgehog* promoter	IV
Wnt1-Cre	(Danielian et al., 1998)	*Cre-recombinase* under *Wingless1* promoter	III
Tal2 null	(Bucher et al., 2000)	null allele of *Tal2*	III
Tal1 Flox	(Hall et al., 2003)	*conditional* allele of *Tal1*	III-IV
Gata2Flox	(Haugas et al., 2010)	*conditional* allele of *Gata2*	I-IV
Gata3Flox	(Kurek et al., 2007)	*conditional* allele of *Gata3*	IV
R26R	(Soriano, 1999)	mouse line carrying inducible *beta galactosidase*	IV
R26R-TdTomato	Jackson laboratories stock number 007194	*Cre reporter strain*; not-recombined cells express red fluorescence and recombined cells express green fluorescence	IV
Gad67^{GFP}	(Tamamaki et al., 2003)	mouse line carrying GFP under the *Gad67* promoter	II-IV

Table 3. Neuronal lineage markers used in this study

Neuronal lineage	Marker	Description	Study
GABAergic neurons	**Gad1/ Gad67**	Glutamate decarboxylase 1, gene encoding enzyme catalyzing GABA from l-glutamic acid	I-IV
GABAergic neurons	**GAD67^{GFP}**	Mouse line expressing green fluorescent protein under Gad67 promoter, marking all the GABAergic neurons.	II-IV
Glutamatergic neurons	**Slc17a6/ Vglut2**	Vesicular glutamate transporter, Mediates glutamate uptake into synaptic vesicles in excitatory glutamatergic neurons	I-IV
Dopaminergic neurons	**TH**	Tyrosine hydroxylase, encoded by Th gene. TH is rate limiting enzyme catalyzing the production of l-dopa from l-tyrosine. L-dopa is precursor of dopamine.	I-IV
Serotonergic neurons	**5-HT**	5-hydroxytryptamine. Monoamine neurotransmitter released by Serotonergic neurons.	I-IV

4. RESULTS AND DISCUSSION

The vertabrate central nervous system contains a great diversity of neurons and glial cells which are generated in the embryonic neural tube at specific times and positions. Several classes of transcription factors have been shown to control various steps in the differentiation of progenitor cells in the neural tube and determine the identity of the cells produced. GABA is principal inhibitory neurotransmitter in the brain. GABAergic neurons play important functions in regulating behavior of an individual and their dysfunction can lead to severe psychiatric and neurological disease. Midbrain GABAergic neurons consist of several anatomical and functional subpopulations, but their molecular and developmental regulatory mechanisms are still not completely understood. We have investigated the molecular mechanisms of GABAergic neurogenesis in diencephalon, midbrain and anterior hindbrain.

4.1 Requirement for Ascl1 during midbrain GABAergic neuron development is region specific (I)

Proneural protein Ascl1 has been shown to be required for GABAergic neuron development in the basal forebrain and midbrain (Casarosa et al., 1999;Miyoshi et al., 2004). The reported complete loss of GABAergic neurons by Miyoshi in the *Ascl1* deficient midbrain was analyzed rather superficially at the beginning of the GABAergic precursor differentiation (Miyoshi et al., 2004). We studied Ascl1 requirement for midbrain GABAergic neuron development in more detail and analyzed several different time points during embryonic GABAergic neurogenesis. In contrast to previous study, we showed that Ascl1 is differentially required for the development of distinct GABAergic neuron subpopulations.

Based on the region specific expression of different transcription factors during the early embryonic stages midbrain can be ventro-dorsally divided into progenitor domains (m7-m1) (Kala et al., 2009;Nakatani et al., 2007). Ultimately, GABAergic neurons arise from ventral m5-m3 and dorsal m2-m1 domains which during the development give rise to different mature midbrain GABAergic subpopulations. The analysis of *Ascl1* deficient midbrain showed that the dorsal midbrain GABAergic neurons had the most stringent requirement for Ascl1, as no GABAergic neurons were generated in the SC, IC and dPAG region of *Ascl1* deficient dorsal midbrain.

The medial midbrain GABAergic neurons which potentially derive from ventral m5-m3 domain showed less dependence on the Ascl1 function. The medial GABAergic neuron subpopulation in the vPAG and MBRf were born in reduced numbers but retained their neurotransmitter identity. In contrast to the dorsal midbrain where Ascl1 was absolutely required for GABAergic neuron development, in the medial midbrain Ascl1 was required for the regulation of GABAergic neuron precursor cell cycle exit and subsequent timing of their production.

Interestingly, Ascl1 was dispensable for the development of the most ventral GABAergic neuron subpopulation in the VTA/SNpr and in the rhombomere1. These GABAergic neurons were also spared in the $Helt^{KO}$ and in the $Gata2^{cko}$ midbrain. In the

latter, all the other midbrain GABAergic neuron subpopulations are transformed into glutamatergic neurons (Kala et al., 2009). In the studies III and IV we showed that the ventralmost GABAergic neurons have developmental origins outside the midbrain and they utilize unique developmental regulatory mechanisms.

4.1.1 Ascl1 has multiple distinct roles in the regulation of GABAergic progenitor proliferation and precursor cell-cycle exit (I)

Recently Ascl1 transcriptional target genes were shown to include genes important for both cell cycle progression and termination and therefore for the entire program of neurogenesis (Castro and Guillemot, 2011;Castro et al., 2011). This might partially explain the diverse effects we detected in the expression pattern of cell cycle regulators between different midbrain domains in *Ascl1*^{KO}. Proneural proteins are known to promote neurogenesis by acting together with cell cycle regulators, specifically with cyclin-dependent kinase inhibitors (Guillemot, 2007).

Interestingly, in the absence of *Ascl1*, we observed ectopic upregulation of negative cell cycle regulators p27 and p57 together with postmitotic cell marker HuC/D specifically in the VZ of the midbrain m4 domain. Consequently, cyclinD1 was downregulated. In contrast, in the flanking m5 and m3 domains, the expression of p27, p57 and HuC/D were reduced in the MZ and proliferative progenitor marker Sox2 was expanded. Therefore, there seemed to be differences in the regulation of progenitor proliferation and differentiation by Ascl1, even within m5-m3 domains. In the m4 domain Ascl1 potentially have a role as a positive cell cycle regulator and under normal circumstances Ascl1 promotes cell cycle progression and proliferation. Subsequently, the *Ascl1* deficient progenitors in m4 precociously activated postmitotic differentiation genes. In contrast, in the m3 and m5 domains, Ascl1 promotes cell cycle exit and regulates negative cell cycle target genes. There the Ascl1 deficient proliferative progenitors were unable to exit the cell cycle at normal temporal pace and neurogenesis was delayed. The reduction of *Gad1* expressing cells in the GABAergic subpopulations of MBRf and vPAG in *Ascl1*^{KO} at later developmental stages was the ultimate outcome of delayed neurogenesis, as the GABAergic precurors in m3 and m5 were born in shorter time window and in reduced numbers.

Proneural genes are well known of their roles in the regulation of lateral inhibition, the phenomenon, where the crucial balance between proliferation and differentiation of neural progenitors is achieved by repressive regulation between proneural genes and Notch signalling (Kageyama et al., 2008). Indeed, studies done in the the basal forebrain showed that loss of Ascl1 leads to region specific downregulation of *Delta1/3* and *Hes5* (Casarosa et al., 1999). Consistently, at early embryogenesis, we observed loss of *Delta1* and *Hes5* expression in the m5-m3 domains of *Ascl1*^{KO}. It has been suggested that proneural protein expression levels are fluctuating during neurogenesis. In proliferating progenitors the oscillatory expression of proneural genes is required for the maintenance of their stem cell characters and later, as neurogenesis proceeds, the proneural protein expression is sustained, promoting neuronal differentiation (Kageyama et al., 2009;Kageyama et al., 2009). Interestingly, we also observed upregulation of bHLH transcription factor Helt in the *Ascl1*^{KO} domains m5-m3. Helt is normally expressed in

salt-and-pepper pattern in the VZ of the developing neuroepithelium. This expression pattern resembles oscillatory expression pattern of Notch pathway members, Delta1 and Hes5. Potentially, *Helt* is normally repressed by Hes5 and the downregulation of Hes5 might cause the ubiquitious expression pattern of Helt in the m5-m3 domains of Ascl1 mutant midbrain. In summary, in the *Ascl1*^{KO} midbrain, the altered expression of cell cycle regulators and lateral inhibition genes showed that the separation and normal balance of proliferation and differentiation fails in the m5-m3 domains which lead to abnormal development of different mature GABAergic nuclei.

Surprisingly, the early loss of *Delta1*, *Hes5 and* Helt expression was restored in the *Ascl1*^{KO} m5-m3 domains two days later. There might be another proneural protein that compensates the loss of *Ascl1*. Nevertheless, we did not observe any ectopic expansion of the proneural genes *Ngn1* or *Ngn2* in the *Ascl1*^{KO} m5-m3 domains. Therefore the candidate molecule that mediates molecular regulation of *Delta1* and *Hes5* and restores their expression in the *Ascl1*^{KO} midbrain remains unsolved. Alternatively, proneural proteins may stimulate but not be absolutely required for GABAergic neurogenesis.

4.1.2 Ascl1 is dispensable for the development of the Ventral Tegmental Area-Substantia Nigra associated and rhombomere1 GABAergic subpopulations (I)

Gata2 has been shown to be crucially important for the development of embryonic midbrain GABAergic precursors and it was previously shown that conditional inactivation of *Gata2* in the midbrain and r1 region leads to fate transformation of dorsal and medial subpopulations of midbrain GABAergic neurons into excitatory glutamatergic ones (Willett and Greene, 2011)(Kala et al., 2009). However, the most ventral GABAergic neuron subpopulation, VTA and SNpr associated GABAergic neurons, are not affected in *Gata2*^{cko} midbrain. Interestingly, our results demonstrated that also without *Ascl1*, the GABAergic neurons associated with dopaminergic nuclei in the ventralmost midbrain and in r1 region of the hindbrain develop normally. This further indicates that the VTA and SNpr GABAergic neurons have different developmental characteristics compared to other midbrain GABAergic subpopulations.

Proneural proteins have multiple roles during neurogenesis which they mediate together with different region specific co-transcription factors (Guillemot, 2007). The ultimate outcome of neurogenesis is to induce different neuronal subtypes at developmentally correct time point and at correct numbers in defined regions of the developing nervous system. Characterization of the Ascl1 targets includes genes involved in cell proliferation, cell specification, differentiation and neurite morphogenesis (Castro et al., 2011;Pacary et al., 2011). It is intriguing that proneural proteins have the capacity to regulate such versatile and distinct steps of neurogenesis. Therefore it seems that Ascl1 participates in all the different steps of neurogenic program. Currently it is not known how Ascl1 regulate and activate such diverse and sequential processes during neurogenesis. However, it is definite that Ascl1 mediate these events with various partners in sequential order and region dependently. In all, the recent reports clarifying the role of Ascl1 in mastering neurogenesis and our observation for the region specific require-

ment of Ascl1 in the midbrain GABAergic neurogenesis and its diversified role in the cell cycle regulation during neurogenesis are very consistent. Based on the phenotypic analysis of both *Ascl1*^{KO} and *Gata2*^{cko} and the comparison of the different transcription factor expressions in these mutants at early and late embryonic stages we propose a model of the specific neuroepithelial regions in developing midbrain giving rise to different mature GABAergic neuron subpopulations (Figure 5).

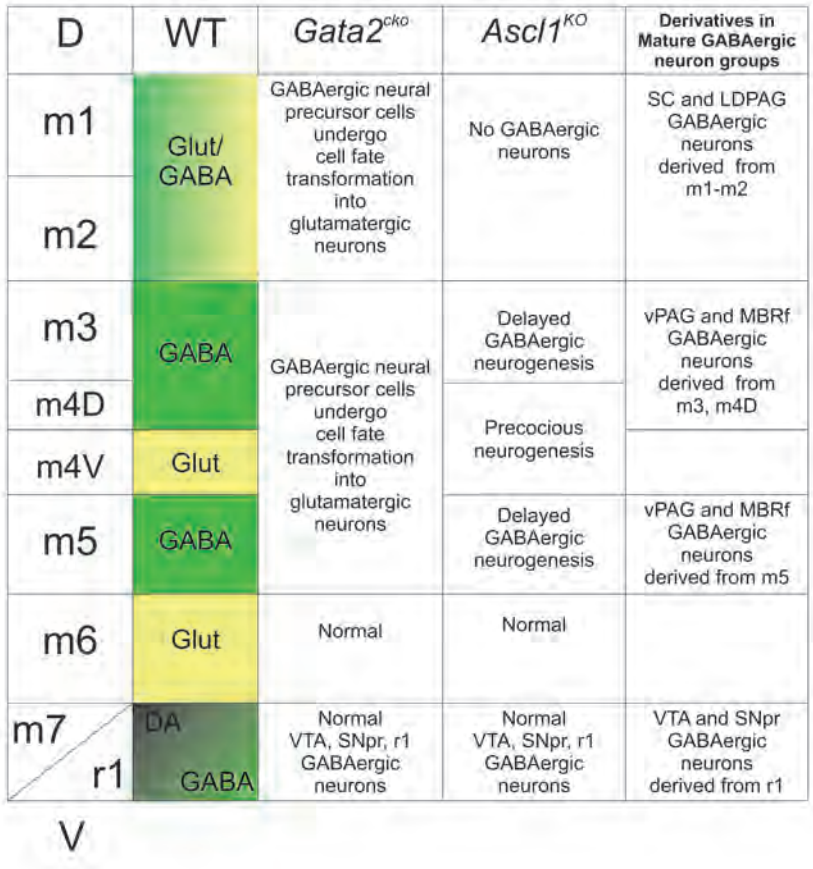


Figure 5. Phenotypic comparison of *Ascl1*^{KO} and *Gata2*^{cko} and proposed model of the specific neuroepithelial regions of developing midbrain giving rise to different mature GABAergic neuron subpopulations. In midbrain domains m1–m2, Ascl1 function is absolutely required as no GABAergic neurons differentiate from Ascl1 deficient m1-m2 domains. In domains m3–m5, inactivation of Ascl1 results in delayed neurogenesis and in m4 precocious activation of postmitotic markers. Unlike in *Gata2*^{cko}, there is no evidence of cell fate transformation in the GABAergic neurons of *Ascl1*^{KO}. GABAergic neurons associated with dopaminergic nuclei in VTA, SNpr and in r1 do not require Gata2 or Ascl1 function. IC, inferior colliculus; MBRf, midbrain reticular formation;LDPAG, dorsolateral periaqueductal gray; SC, superior colliculus; SNpr, substantia nigrapars reticulata; r1, rhombomere 1; vPAG, ventrolateral periaqueductal gray; VTA,ventral tegmental area

4.2 Gata2 and Ascl1 regulate GABAergic neurogenesis, GABAergic neurotransmitter and subtype identity in the developing diencephalon (II)

During development the diencephalon is subdivided into three different domains, prosomeres; P1, P2 and P3. Although it is known that GABAergic neurons are born in restricted domains of P1, rostral P2 (p-Th-R) and P3, the molecular regulation of different GABAergic neuron populations development in distinct prosomeres is still not completely understood. Ascl1 is expressed in all the prosomeres giving rise to GABAergic neurons in the diencephalon (Kataoka and Shimogori, 2008;Vue et al., 2007). Previously, it was reported that in the *Ascl1* deficient diencephalon, the GABAergic neuron development is lost posterior to Zli (Miyoshi et al., 2004). However, as in the midbrain, the authors did not thoroughly investigate the region specific differencies of Ascl1 function during diencephalic GABAergic neurogenesis.

Another important transcription factor for GABAergic neurogenesis, Gata2 is also expressed in the diencephalon, although the expression pattern differs from Ascl1. In our studies we showed that Gata2 is expressed in the postmitotic GABAergic domains of the P1 and rostral P2 (pTh-R) but is excluded from P3. We analyzed Ascl1 and Gata2 requirement for the GABAergic neuron development in diencephalon and revealed that Gata2 and Ascl1 are differentially required for GABAergic neurogenesis between distinct diencephalic prosomeres. Most importantly, our studies are the first demonstrations that *Gata2* acts as a post-mitotic selector gene during GABAergic neurotransmitter identity selection in P1 and subtype identity selection in pTh-R (Figure 6).

4.2.1 Ascl1 is differentially required for GABAergic neurogenesis in the P1, pTh-R and P3 of the developing diencephalon (II)

To gain insight into how Ascl1 is required for diencephalic GABAergic neuron development, we analyzed GABAergic and glutamatergic marker expressions in the developing *Ascl1* deficient diencephalon. Our studies showed that Ascl1 differentially regulates neurotransmitter identity formation and progenitor proliferation in distinct GABAergic neuron progenitor populations.

In P1, we observed complete loss of GABAergic neurons and subsequent upregulation of *Ngn2* and ectopic glutamatergic neuron production. In order to activate downstream target genes important for neuronal subtype specification, proneural genes are known to cross-repress each other. Otx2 mediates *Ascl1* repression and this repression is essential for glutamatergic neuron production from Ngn2+ progenitors in thalamus (Parras et al., 2002;Puelles et al., 2006). Therefore, it seems that Ascl1 is required for *Ngn2* repression and subsequent promotion for GABAergic neuron identity in the P1. We also observed downregulation of Gata2 in the $Ascl1^{KO}$ P1 region. Nevertheless, the transformation of the progenitor identity can be expected to lead transformation of the later produced post-mitotic precursor identity, and therefore the loss of Gata2 in the *Ascl1* deficient P1 region was not surprising. Interestingly, GABAergic neuron neurotransmitter identity was not affected in the pTh-R and P3 regions. Instead, we observed differential expression of cell cycle regulators in adjacent GABAergic progenitor regions. The

recent findings of the multiple roles of the Ascl1 in neuronal progenitor proliferation and differentiation regulation (Castro and Guillemot, 2011;Castro et al., 2011) prompted us to further analyse the expression of genes characteristic for proliferative progenitors and post mitotic precursors, as well as the cell cycle regulators; cyclin dependent inhibitors p27 and p57 and cyclinD1.

In P1 and P3 the proliferative progenitor layer, positive for Sox2, was significantly thicker when compared to wild-type. Simultaneously, the expression of postmitotic marker HuC/D was downregulated indicating that without Ascl1, the proliferative progenitors in P1 and P3 were not able to exit cell cycle at normal rate. Therefore, it seems that in P1 and P3 Ascl1 promotes cell cycle exit. Interestingly, in pTh-R, we observed upregulation of post-mitotic markers HuC/D and p27 and concurrent downregulation of CyclinD1 indicating that in pTh-R Ascl1 is required for promotion of cell cycle progression. Therefore, our results show that Ascl1 can mediate very diverse and even opposite roles in the distinct diencephalic GABAergic progenitor populations. The differential outcome of the defects seen in distinct diencephalic domains of *Ascl1*^{KO} indicates that Ascl1 activates diverse cell cycle target genes in these domains. This was also seen in the midbrain, where Ascl1 differentially regulates progenitor proliferation and differentiation in adjacent midbrain domains (See 4.1.1).

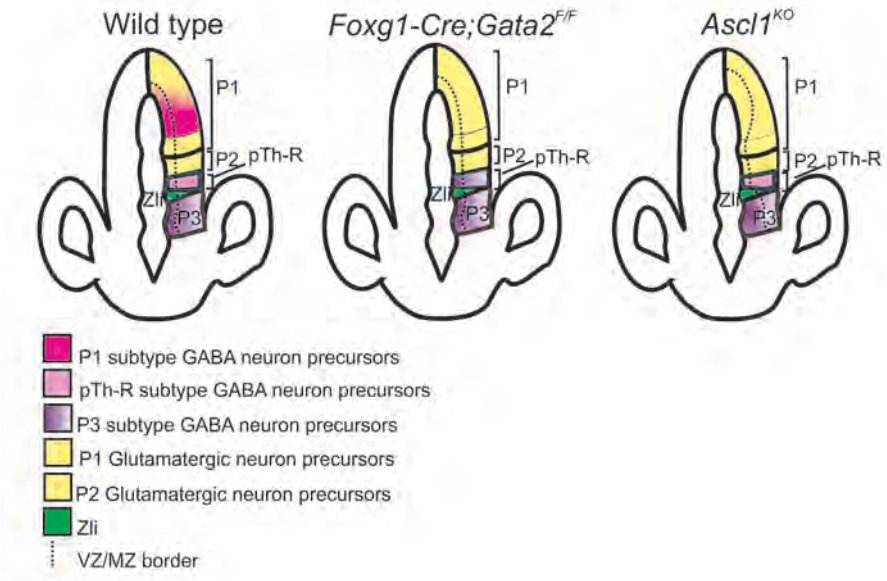


Figure 6. Schematic representation of Gata2 and Ascl1 regulated GABAergic neurogenesis and prosomeric identity specification during the early development of diencephalon. Coronal sections representing E12.5 embryos diencephalic P1-P3 regions. GABAergic neurons are generated in the P1, pTh-R and P3 regions. *Ascl1* inactivation leads to complete loss of GABAergic neurons in P1 region. Neither GABAergic neuron neurotransmitter nor prosomere subtype identity was affected in the *Ascl1* deficient pTh-R and P3 regions. Based on the expression of cell cycle regulators Ascl1 promotes cell cycle exit P1, P3 and prevents it in pTh-R. Loss of *Gata2* leads to neurotransmitter identity change in P1. In pTh-R, the *Gata2* deficient GABAergic neuron precursors adapt P3 specific GABAergic neuron phenotype.

4.2.2 Gata2 specifies GABAergic neurotransmitter identity in P1 and GABAergic neuron subtype identity in pTh-R (II)

As proliferating progenitors leave the cell cycle they start to express neuron specific genes that define neuronal characteristics important for the function of a terminally differentiated neuron. Terminal selector genes are activated in postmitotic cells where they activate downstream target genes, terminal differentiation genes crucial for ultimate identity of a fully functional neuron. In the case of inactivation of terminal selector genes, the neuron specific downstream differentiation genes are not activated. This generally has devastating consequences on the differentiation program of a neuron. Typically this results in either the loss or identity switch of a specific neuronal subtype (Hobert, 2008)(Cheng et al., 2004;Liu et al., 2010).

At early stages of neurogenesis, the inactivation of *Gata2* in the diencephalon led to the downregulation of GABAergic neuron specific genes and subsequent neurotransmitter identity change of P1 GABAergic neurons into glutamatergic ones. Surprisingly, the ectopic expression of *Slc17a6* was restricted only in the P1. In contrast, the pTh-R GABAergic neuron precursors responded differentially to the loss of Gata2. There, pTh-R specific GABAergic neuron markers *Gata3, Tal1* and *Npy* were downregulated but the post-mitotic cells retained GABAergic identity. Interestingly, the GABAergic neuron precursors started to express P3 specific GABAergic markers, *Arx* and *Dlx*, indicating that the loss of *Gata2* led to prosomeric GABAergic neuron subtype identity switch of pTh-R precursors into P3 specific neuron precursors. Our results demonstrate that, without *Gata2* the P1 precursors undergo neurotransmitter fate change from GABAergic to glutamatergic, but the pTh-R GABAergic precursors (normally $Gata3^{+}/Tal1^{+}/Npy^{+}$) activate P3 specific genes and adopt characteristics of more rostral GABAergic phenotype.

4.2.3 Neurotransmitter and subtype identity switch is maintained in the $Ascl1^{KO}$ and $Foxg1\text{-}Cre^{+};Gata2^{Flox/Flox}$ perinatal diencephalon (II)

The thalamic neuronal nuclei can be anatomically subdivided into three different populations: prethalamus, thalamus and pretectum. Glutamatergic neurons are mainly populating the thalamic nuclei, in contrast the GABAergic nuclei locate in the pretectal and prethalamic area (Figure 7). Located near the prethalamic and thalamic boundary are the important GABAergic nuclei, the ventral lateral geniculate nucleus (vLGN) and intergeniculate leaflet (IGL). We mapped the expression of pTh-R specific GABAergic markers *Gata3, Tal1, Nkx2.2, Npy* and *Penk* and P3 specific GABAergic markers *Arx* and *Dlx* in the perinatal diencephalon and compared the expression patterns to the glutamatergic markers *Slc17a7* and *Ngn2*. *Gata3* and *Tal1* were expressed in the pretectal area and are likely derived from P1 GABAergic domain. *Gata3* was uniformly expressed in the IGL together with other pTh-R specific genes *Npy, Penk.* In lateral vLGN there was abundant expression of *Gata3, Penk, Tal1* and *Nkx2.2.* All the glutamatergic and prethalamic nuclei were negative for Gata3 expression. Based on the restricted expression of genes normally expressed in P1 and pTh-R, such as *Gata3/Tal1* and other GABAergic markers, we hypothesized that the mature GABAergic nuclei in APN, IGL and lateral vLGN

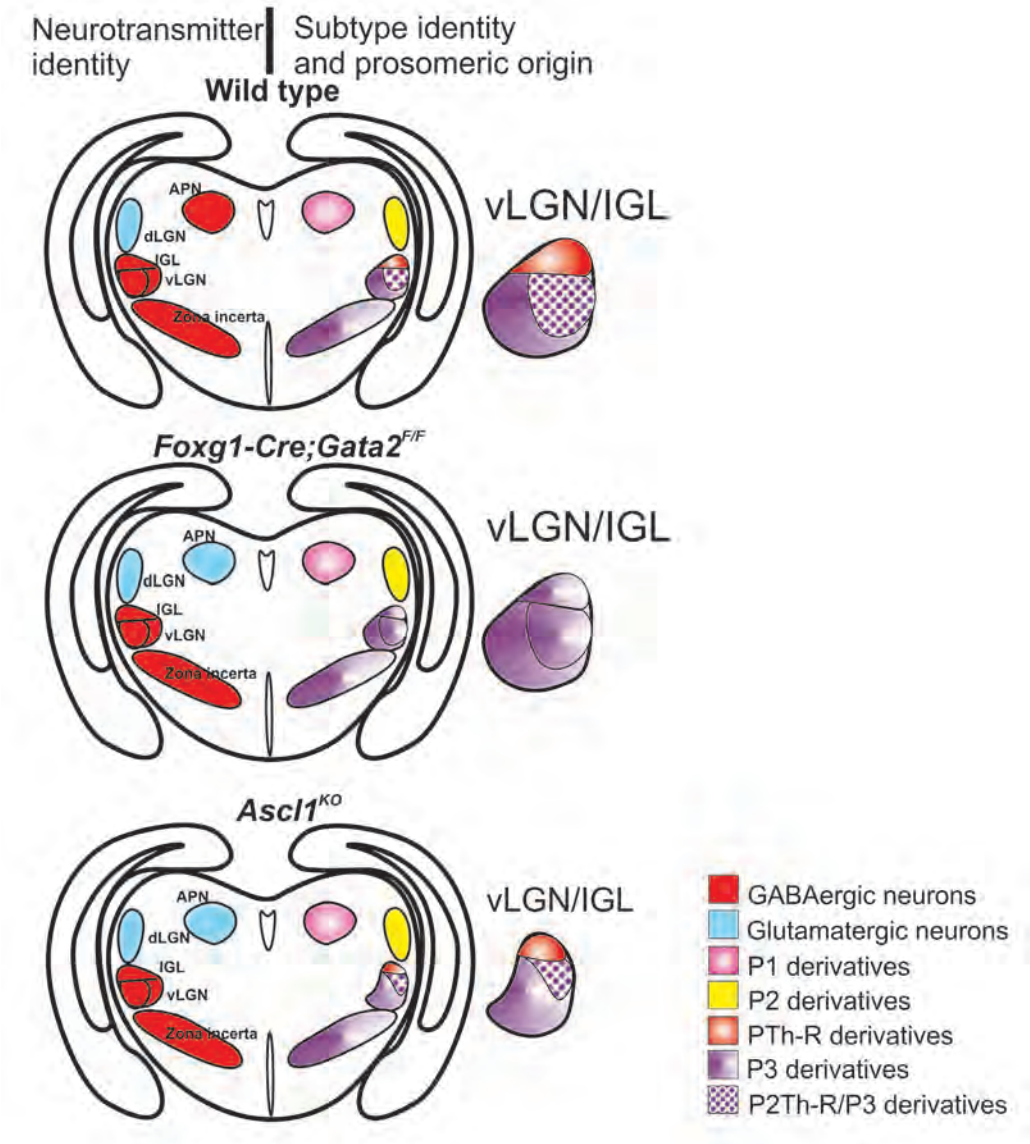


Figure 7. Proposed schematic representation of different diencephalic neuronal nuclei and their neurotransmitter identity and potential prosomeric origins in *Wild-type*, *Foxg1-Cre;Gata2*$^{F/F}$ and *Ascl1*^{KO}. GABAergic neuronal nuclei include the anteropretectal nucleus (APN), intergeniculate leaflet (IGL), ventrolateral genigulate nucleus vLGN and Zona incerta. Based on differential expression of GABA neuron markers in distinct prosomeres, the APN GABAergic neurons are potentially descendants of P1 GABAergic neuron precursors, IGL GABAergic neurons are pTh-R derivatives and vLGN GABAergic neurons derive both from pTh-R and P3 precursors. Zona incerta GABAergic neurons are P3 derivatives and dorsal lateral genigulate nucleus glutamatergic neurons are derived from caudal part of P2. The defects in GABAergic neuron identity development in early stages were persistent in both *Foxg1-Cre;Gata2*$^{F/F}$ and *Ascl1*^{KO} until birth. (Modified from Pro-Gradu Thesis of Sini-Maaria Virolainen).

are likely derivatives of P1 and pTh-R. P3 specific genes *Arx* and *Dlx* showed restricted expression in the medial part of vLGN.

The analysis of perinatal brains demonstrated that the defects in GABAergic neuron identity development in both *Ascl1*^{KO} and *Foxg1-Cre*$^{+}$*;Gata2* $^{Flox/Flox}$ were persistent at birth. In the *Ascl1* deficient diencephalon, the P1 derived GABAergic neurons of APN were abundantly expressing glutamatergic marker *Slc17a6*. Therefore, the loss of Ascl1 led to complete failure of GABAergic neuron development in P1 and the derived structures, and this defect was persistent until birth. Consistent with the embryonic phenotype, IGL/vLGN, the potential derivatives of pTh-R and P3, contained substantial *Gad1*+ neurons, although the nuclei size and organization was altered. In the *Ascl1*^{KO} midbrain, as a result of delay in the neurogenesis in the m5-m3 domains, the GABAergic neuron derivatives of m5-m3 are born in reduced numbers. Possibly, similar mechanism result the reduction in the size of IGL/vLGN nuclei which are likely the pTh-R/P3 GABAergic neuron derivatives. In conclusion, in contrast to P1, Ascl1 is not required for definition of GABAergic neuron identity or for GABAergic neuron subtype identity specification in the pTh-R and P3 derived structures but is required for production normal amount of neurons (See Figure 7).

The expression of *Gad1* and *Gata3* was abolished from APN pretectal area and was replaced with abundant expression of glutamatergic marker *Slc17a6* also in *Foxg1-Cre*$^{+}$*;Gata2* $^{Flox/Flox}$ indicating that the neurotransmitter fate change from GABAergic to glutamatergic neurons seen in P1 was persistent until perinatal stages. The IGL/vLGN of *Foxg1-Cre*$^{+}$*;Gata2* $^{Flox/Flox}$ contained an apparently normal amount of *Gad1* expressing cells. However, we did not detect expression of *Gata3, Tal1 or Npy*. Instead, the P3 specific GABAergic markers *Arx* and *Dlx* were now expressed in the IGL. Therefore, it seems that during diencephalic GABAergic neurogenesis, Gata2 not only specifies neurotransmitter identity but it is also required for the prosomere specific identity of differentiating GABAergic neurons (See II, Figure 8 and this thesis work Figure 7).

4.3 Distinct functions of Gata2 co-factors Tal1 and Tal2 in the development of midbrain GABAergic neurons (III)

The VTA-SN associated GABAergic neurons were spared in the *Gata2*^{cko} and *Ascl1*^{KO} midbrain suggesting they have unique characteristics in their gene expression profile and developmental pathways ((Kala et al., 2009), I). These studies showed that there are developmental differences between distinct GABAergic neuron subpopulations in the midbrain.

Gata2 cofactors, Tal1 and Tal2, were previously shown to be expressed in the CNS during the embryonic development. Although conditional inactivation of *Tal1* (Bradley et al., 2006) and null deletion of *Tal2* (Bucher et al., 2000) led to abnormalities in the brain development, the detailed cell type specificity and developmental mechanisms were not addressed by these studies. Gata and Tal transcription factors have important roles during hematopoietic development. In the hematopoietic system and in the spinal cord they have been shown to form multiprotein transcription regulatory complexes in order to control transcriptional regulation of target genes (Joshi et al., 2009;Wadman et

al., 1997). As GATA-Tal transcriptional regulatory complexes operate in other regions of developing embryo; we were interested on the requirement of Gata-Tal transcription factor functions for GABAergic neuron development in the midbrain and r1.

To demonstrate the requirement of GATA and Tal factors for GABAergic neuron differentiation in the midbrain and rhombomere1, we used mouse lines, where the function of *Tal2* (*Tal2*^{ko}), *Tal1* (En1^{cre}; Tal1$^{Flox/Flox}$ = *Tal1*^{cko}) or both *Tal1/2* (Wnt1^{cre};Tal1$^{Flox/Flox}$; Tal2^{KO} = *Tal1/2*^{dko}) were eliminated. By comparing *Tal* mutant mouse lines with the *Gata2* mutant mouse line (En1^{cre}; Gata2$^{Flox/Flox}$ = *Gata2*^{cko}) we were able to elucidate the combinatorial actions of these factors during GABAergic neuron development in midbrain and r1.

4.3.1 Gata2 and Tal2 are both required for midbrain GABAergic neuron development

Our results revealed that *Tal1, Tal2, Gata2* and *Gata3* were expressed in the GABAergic domains of developing midbrain and r1 region. However, the expression patterns of *Tal1* and *Tal2* were different between the midbrain and r1. We showed that both Tal1 and Tal2 are important for GABAergic neuron differentiation. However, the requirement for their function is region specific and consistent with the expression profile and intensity between the midbrain and r1.

In the midbrain *Tal2* expression preceded *Tal1*, and *Tal2* was co-expressed with *Gata2* in the VZ and early differentiating GABAergic neurons of IZ. In the r1, *Tal1* expression preceded *Tal2* and was expressed in the VZ and IZ of GABAergic neuron precursors.

The analysis of the *Gata2*^{cko}, *Tal2*^{ko} and *Tal1/2*^{dko} embryos during early midbrain GABAergic neurogenesis showed that *Tal1* and *Tal2* are differentially required for GABAergic neuron precursor differentiation between midbrain and r1. The inactivation of *Tal2* led to defect in the activation of complete set of GABAergic target genes and subsequent fate transformation of differentiating GABAergic cells into glutamatergic neurons in the midbrain m5-m3 domains. In contrast, Tal1 was specifically required for GABAergic neuron precursor differentiation in the ventral rhombomere1 but not in the midbrain.

The requirement for Tal2 in the GABAergic neuron differentiation is very similar to Gata2, which regulates the postmitotic specification of GABAergic neuron precursors in the posterior diencephalon and in the dorsal and medial midbrain (II, (Kala et al., 2009)). *Tal2* and *Gata2* were independently activated in midbrain. The exact mechanism behind their activation is currently not known. They are both activated immediately after the cell cycle exit and their activation is potentially dependent on the cell cycle phase and other region specific molecules. Their inactivation in the midbrain did not affect progenitor cell proliferation, cell cycle exit, or neuronal precursor production. Although, the phenotypes of *Gata2*^{cko} and *Tal2*^{ko} resemble each other, we noticed some differences between them. In *Tal2*^{ko} midbrain there were still some *Gad1* expressing GABAergic neurons produced in the m5 domain. Additionally, there seemed to be differences in the activation of other GABAergic specific markers genes. Therefore, both Gata2 and Tal2

potentially have functions independent of each other. However, they are both required for the complete activation of midbrain GABAergic differentiation.

In summary, we suggest that specifically in the midbrain, simultaneous expression of Gata2 and Tal2 and subsequent formation of GATA2/Tal2 transcription regulatory complex regulates genes crucial for postmitotic differentiation of GABAergic neuron precursors and their identity specification (Figure 9, III Figure 8).

4.3.2 Cellular neurotransmitter fate change is persistent to perinatal stage in the Tal1/2 and GATA2 mutant midbrains

The defects seen in prenatal stages were persistent throughout embryological development until birth. GABAergic and glutamatergic neuronal nuclei can be divided into different anatomical and functional subpopulations in the midbrain. GABAergic nuclei in the midbrain dorsal, medial and ventral subpopulations express *Gad1, Gata3* and *Tal1*. In addition, the most ventrolateral population of near SN also expresses *Six3* and *Sox14*. Consistent with the early phenotype, the GABAergic neuron marker genes were downregulated in the dorsal midbrain of $Tal2^{ko}$ and $Tal1/2^{dko}$. In the medial midbrain (mRF) few scattered cells were still expressing *Gad1* and *Gata3* which were potentially descendants of cells expressing *Gata3+* in the m5 domain during prenatal stage (Our unpublished results). We compared the GABAergic marker gene expression pattern of $Tal2^{ko}$ and $Tal1/2^{dko}$ to the $Gata2^{cko}$, in which all the other GABAergic neuronal nuclei except the most ventral VTA/SNpr area had lost the expression of *Gad1* and *Tal1*. Both *Gata2* and *Tal1/2* mouse mutant lines showed robust increase in the glutamatergic neuron marker *Slc17a6* expression in the areas where GABAergic neurons were lost. However, the VTA-SNpr GABAergic neurons behave very differentially.

Together, our results demonstrate that the cellular neurotransmitter fate change of GABAergic neurons in the dorsal and medial midbrain into glutamatergic ones was persistent from prenatal to perinatal stages in all analysed mouse lines. The most ventral subpopulation of GABAergic neurons were still retained in the $Gata2^{cko}$ and $Tal2^{ko}$ midbrain, indicating that this subpopulation utilizes very different developmental regulatory mechanisms compared to other midbrain GABAergic subpopulations.

Tal1 is normally expressed by the VTA/SNpr and r1 GABAergic neurons. The development of GABAergic neurons in these brain regions is not regulated by transcription factors Ascl1 and Gata2 which are known to be required for the development of dorsal and medial GABAergic neuron subpopulations (Kala et al., 2009; I). Interestingly, the inactivation of both *Tal1* and *Tal2* factors in the midbrain and r1 led to complete loss of all the midbrain GABAergic subpopulations including VTA/SNpr GABAergic neurons at birth (unpublished results). Therefore to clarify the mechanisms in the regulation of VTA/SNpr GABAergic neurons we analyzed GABAergic neurogenesis more carefully in the closely situated brain region, rhombomere1.

4.3.3 Domains of GABAergic neurogenesis in r1 (III, IV)

The cell type specificity of Tal1 and Tal2 expression in the brain has not been previously demonstrated. In order to characterize GABAergic neurogenesis in detail in the r1, we delineated a map of different domains in the ventrolateral r1giving rise to GABAergic neural precursors. We compared the expression of *Gata2, Gata3, Tal1* and *Tal2* to GABAergic and regional markers in the r1. *Gata2, Gata3* and *Tal1*were expressed in the same specific ventromedial domain of r1. We also detected weak expression of *Tal2* in this domain. The ventromedial *Gata+/Tal+* domain was positive for homeobox transcription factor Nkx6.1. *Gata2* and *Gata3* are also expressed in the serotonergic 5-HT+ neurons. In contrast, we could not detect *Tal1 or Tal2* expression in the ventral 5-HT^{+} domain. Another *Gad1+* but *Gata* and *Tal* negative domain was located in the lateral Nkx6.1 negative region. These GABAergic precursors potentially give rise to cerebellar GABAergic neurons as they express bHLH transcription factor *Ptf1a* which has been shown to be the main driver of GABAergic neurogenesis in the cerebellum (Hoshino et al., 2005). Therefore, we identified distinct GABAergic domains with unique transcription factor expression pattern in the ventrolateral r1 (Figure 8).

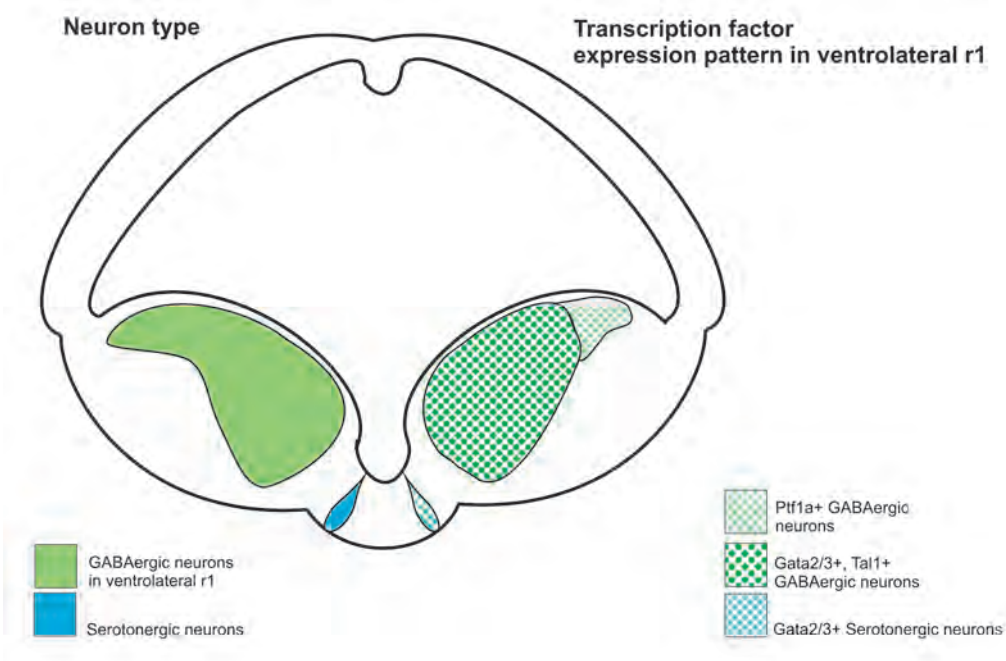


Figure 8. Schematic representation of E12.5 transverse section representing ventral r1 GABAergic and serotonergic neuronal domains. *Gata2, Gata3* and *Tal1* are expressed in the ventromedial r1 domain (dark green spheres). This ventromedial domain is Nkx6.1+. *Gata2* and *Gata3* are expressed in the Serotonergic neurons but *Tal1* and *Tal2* are not. *Ptf1a+* GABAergic domain (light green spheres) is located in the ventrolateral r1 and is negative for *Gata2/3* and *Tal1*.

4.4 Development of VTA/SNpr GABAergic neurons (III,IV)

Although the inactivation of *Tal1* alone in the midbrain did not affect GABAergic neuron differentiation during early embryonic development, we observed loss of the VTA/SN associated GABAergic neuron populations in *Tal1*^{cko} mutants at birth.The ventralmost subpopulation of GABAergic neurons are closely associated with dopaminergic neurons in the VTA and SN. These dopaminergic cells have very important functions in the regulation of voluntary movements and behaviour of an individual and degeneration or abnormal function of these cells has been associated with several neurological diseases. Despite the intensive molecular and developmental regulatory mechanisms characterization of the midbrain dopaminergic neurons, the neighbours, the VTA-SNpr GABAergic neurons, have gained little attention. These DA associated GABAergic neurons convey inhibitory input to SN-VTA dopaminergic neurons and regulate their activity. Therefore, it is of special interest to reveal the molecular regulation, developmental and migratory pathways of VTA-SN associated GABAergic neurons.

The VTA/SNpr associated GABAergic neurons are spared in *Gata2* and *Ascl1* deficient and lost in the *Tal1*^{cko} and *Tal1/2*^{dko} midbrain (Kala et al., 2009)I,III). In addition, *Gata2* and *Ascl1* are strongly expressed in the posterior diencephalon and r1. We hypothesized that VTA/SNpr GABAergic neurons might potentially originate from the adjacent brain regions, diencephalon and r1. We utilized Cre-recombinase based genetic fate mapping analyses and tissue specific inactivation of *Gata2, Gata3* and *Tal1* in order to elucidate more carefully the origin of the midbrain GABAergic neurons.

4.4.1 Experimental set up of the genetic fate mapping by Cre-recombinase (IV)

For Cre-recombinase based genetic fate mapping we crossed mouse lines carrying tissue specific Cre-recombinase with reporter mice carrying reporter alleles *R26R* and *TdTomato*. Subsequently upon Cre mediated site-specific recombination the reporter alleles permanently expressed b-galactosidase (b-gal) (R26R) or red fluorescent protein (RFP) (TdTomato). By using different Cre-lines, we were able to identify cells originating from different brain compartments. *En1-Cre* mediated recombination labelled cells originating from posterior diencephalon, midbrain and rhombomere1, *Shh-Cre* labelled cells originating from the ventral floor plate and in the midbrain the ventral and lateral floorplate. The third Cre-line, *Gbx2-Cre*^{Ert2} labelled cells originating from r1 and ventral midbrain floor plate. *Gad67GFP* allele which produces GFP in all Gad1 expressing cells was crossed to reporter mouse lines to facilitate the identication of GABAergic neurons.

4.4.2 Substantia Nigra pars reticulata GABAergic neurons can be divided into two cell populations with distinct origins (IV)

Genetic fate mapping demonstrated that the midbrain VTA/SNpr GABAergic neurons are derived from *Gbx2*^{CreERT2} labelled cell lineage in the r1. Comparing labelling patterns

of three different mouse lines; *En1*^{Cre}, *Shh*-^{Cre} and *Gbx2*^{CreERT2}, we were able to deduce distinct origins of midbrain GABAergic neurons. *En1*-^{Cre} labelled all the midbrain cells, in addition the anterior part of SNpr in the P1 region of diencephalon. *Shh*^{Cre} specifically labelled the cells that give rise to the midbrain dopaminergic cells and the red nucleus neurons. Gbx2 is normally expressed in the ventral midline of the midbrain and in the r1. With the comparison of *Shh*-^{Cre} and *Gbx2*-^{CreERT2}labeled cells, we were able to elucidate the origin of VTA/SNpr GABAergic neurons in the r1 region of the hindbrain. Interestingly, based on the origin of the SNpr GABAergic neurons, SNpr can be divided into two domains: anterior diencephalic and posterior midbrain SNpr. The diencephalic anterior SNpr was labelled only with *En1*^{Cre} , in contrast, the posterior SNpr was additionally labelled with *Gbx2*^{CreERT2}. Furthermore, we showed that the anterior and posterior SNpr domains have differencies in their requirement for midbrain GABAergic neuron promoting transcription factor Gata2. The diencephalic anterior SNpr GABAergic neurons were lost in the *Foxg1*^{Cre}*; Gata*^{FloxFlox} mutants and therefore this GABAergic neuron precursors are dependent on the Gata2 function in order to develop normally. Interestingly, the posterior midbrain parts of SNpr GABAergic neurons do not require any of the general GABA promoting transcription factors Ascl1, Helt or Gata2 for their development (Guimera et al., 2006)(Kala et al., 2009)(Peltopuro et al., 2010). In the *Gata2*^{cko} midbrain, the VTA-SN associated GABAergic population is not affected although all the other midbrain GABAergic populations loose their identity and transform into glutamatergic neurons. It seems that compared to other midbrain GABAergic neuronal populations, the VTA-SNpr GABAergic neurons have unique developmental mechanisms and they potentially originate from adjacent brain regions.

4.4.3 Tal1 controls GABAergic neuron identity in the anterior rhombomere 1 and development of VTA/SN GABAergic neurons (III,IV)

The *Gata2*^{cko} and *Ascl1*^{KO} phenotypes together with genetic fate mapping experiments suggested that VTA-SN associated GABAergic neuron population might have developmental origins outside the midbrain. Based on the differential transcription factor expression, rhombomere 1 can be subdivided into different subdomains giving rise to GABAergic neurons (Hoshino et al., 2005). We identified *Tal/2+*, *Gata2/3+* subdomain in the ventrolateral r1 producing GABAergic neurons (Figure 8). Compared to midbrain, Gata2 and Tal factors were very differently required for GABAergic neuron development in the ventral r1. Consistent with its expression intensity, *Tal1* was acting as a post-mitotic selector gene specifically in the r1. In the midbrain *Tal1* alone was not required for GABAergic neurogenesis during the early embryological development.

Tal1 mediated regulation of GABAergic neuron identity differentiation in r1 was evident as the normally *Gad1* expressing neural precursors of ventral r1 were substituted with *Slc17a6+* glutamatergic neurons at early embryogenesis and the defect was persistent until birth. In contrast to midbrain, Tal2 was not required for GABAergic differentiation in r1. Whether Tal1 mediates GABAergic neuron identity specification in r1 alone or together with additional factors such as Gata2 and Gata3 remains to be elucidated. Both Gata2 and Gata3 are expressed in the ventrolateral subdomain but neither is alone

required for GABAergic neuronal differentiation in this brain region. On the contrary to Tal1/2, Gata2/3 are expressed in the serotonergic neurons located ventral to *Tal/2+* domain. Previously Gata2 and Gata3 have been shown to be involved in the neuronal differentiation of serotonergic neurons (Craven et al., 2004)(van Doorninck et al., 1999). It will be intriguing to characterize which transcription factors mediate serotonergic neuronal differentiation together with Gata factors. Based on our studies, it is evident that Tal factors are not required for their differentiation.

Altogether, our results suggest that the inactivation of *Tal1* in r1 leads to GABAergic to glutamatergic neuron fate change already at early stages of development and the transformation is persistent until birth. The defect in GABAergic neurogenesis in r1 correlates with a loss of VTA-SN associated GABAergic neurons in the ventral midbrain.

In summary, we propose that similar to the midbrain there is analogous yet molecularly distinct GATA/ Tal transcription regulatory complex regulating GABAergic differentiation in the r1. This complex might take part in the molecular interplay important for correct VTA-SN GABAergic neuronal profile development from the GABAergic neuronal precursors born in r1 (Figure 9).

4.4.4 Migration of the VTA-SN area GABAergic neurons from the r1 (IV)

The BrdU labelling analyses showed that the different midbrain GABAergic neuron progenitor subpopulations complete neurogenesis at different time points. Post-mitotic GABAergic precursors were produced first (at E10.5) at ventral midbrain derived mRF and they completed neurogenesis at E12.5.The SNpr and SC post-mitotic GABAergic neuron precursors were produced later at E11.5 and neurogenesis was completed by E13.5-14.5, respectively. Importantly, at the same time when VTA/SNpr GABAergic neurons are born (E13.5), the $Gata2^{cko}$ midbrain did not express Gad1. However, later in the development, the VTA/SNpr GABAergic neurons reappeared in the ventral midbrain of $Gata2^{cko}$ midbrain and expressed Gad1. These analyzes implied that the posterior VTA/SNpr GABAergic neurons migrate to their final position during the end of embryonic development. Consistently, the first Gad1+ and RFP/$Gad67^{GFP+}$ cells emerged between E14.5 and E15.5 in Wt midbrain. Furthermore, the first $Gad67^{GFP+}$ cells emerged also at between E14.5-15.5 in the $En1^{Cre};Gata2^{flox/flox}$, $Gad67^{GFP}$midbrain. This result together with the genetic fate mapping analyzes support the view of the VTA-SNpr GABAergic neurons arise from r1 region and migrate to ventral midbrain as post-mitotic precursors.

Interestingly, the DA associated GABAergic neurons in the VTA-SNpr region were lost in the $Tal1^{cko}$ midbrain. The inactivation of *Tal1* in the midbrain and r1 leads to neurotransmitter identity change of r1 derived prospective VTA-SNpr GABAergic precursors which subsequently may be incompetent to migrate to their final positions in the ventral most midbrain.

In the forebrain, the tangential migration of GABAergic neuron precursors from ventral to basal forebrain and cortex is complex process which is regulated by several different transcription factors, cell surface receptors and chemical cues. Migrating cor-

tical GABAergic neurons follow specific tangential routes to their final positions and travel across regional forebrain boundaries (Marin and Rubenstein, 2003). The developing nervous system is divided into specific boundary regions which function as organizing centers and express boundary specific transcription factors important for regional patterning. In these boundaries there are specific progenitor cells that behave differentially compared to progenitors located further from boundaries. Close to the boundaries the progenitor cells proliferate slowly and prevent cell movements across boundaries and therefore enhance the compartmentalization of CNS. It has been shown that *Hes* genes are important for boundary formation and are continuously expressed in several boundary regions (Baek et al., 2006). It is generally believed that progenitor cells in VZ respect these boundary regions and do not move across them. However, the post-mitotic precursors in the marginal zone may be less restricted.

In the forebrain, the immature GABAergic neuron precursors travel long distances and across different subdivisions of developing forebrain (Marin and Rubenstein, 2003). Compared to extensively studied forebrain GABAergic neuron migration, very little is known about the molecular regulation of midbrain and r1 GABAergic neuron precursor migration. Complex regulation of forebrain GABAergic neuron migration includes movement stimulating and structural factors together with directional cues from the environment (Marin and Rubenstein, 2003). In the basal forebrain dopamine receptor D1 activation in dopamine rich mileu surrounding GABAergic interneuron precursors induces their tangential migration from basal forebrain to cortical wall (Crandall et al., 2007). Furthermore, in developing interneurons expressing low levels of KCC2 GABA mediates depolarization. This GABA-A receptor mediated depolarizations induce interneuron migration from basal forebrain to cortex. Once GABAergic interneurons reach their destination in the cortex, upregulation of KCC2 in their cell surface leads to hyperpolarization and migration termination (Bortone and Polleux, 2009). It is particularly intriguing to speculate that similar molecular mechanisms might operate in the GABAergic interneuron migration between r1 and VTA-SN area of the midbrain. In the future it will be a special interest to reveal the migratory routes and regulatory mechanisms of clinically important VTA-SN associated GABAergic neuron precursors. It remains to be elucidated, whether they express related molecules and utilize similar mechanisms as the forebrain GABAergic neuron precursor partners.

Our studies are the first demonstrations of the genetic regulatory mechanisms of GABAergic neuron differentiation in clinically significant brain regions. In the midbrain VTA-SN area, the GABAergic neurons convey inhibitory input to VTA-SN dopaminergic neurons and regulate their activity. There are important GABAergic neurons in the ventral midbrain, in the VTA and rostromedial tegmental nucleus (RMTg) which have been shown to be important in the regulation of behavioural responses of an individual. In a mature brain GABA mediated inhibition decrease dopamine release in the midbrain which subsequently influences motivational behaviour of an individual. Currently, the projection characteristics and genetic regulation of RMTg GABAergic neurons are poorly understood. Based on the VTA-RMTg functional relevance for dopamine pathways it could be a fascinating target for advanced drug development.

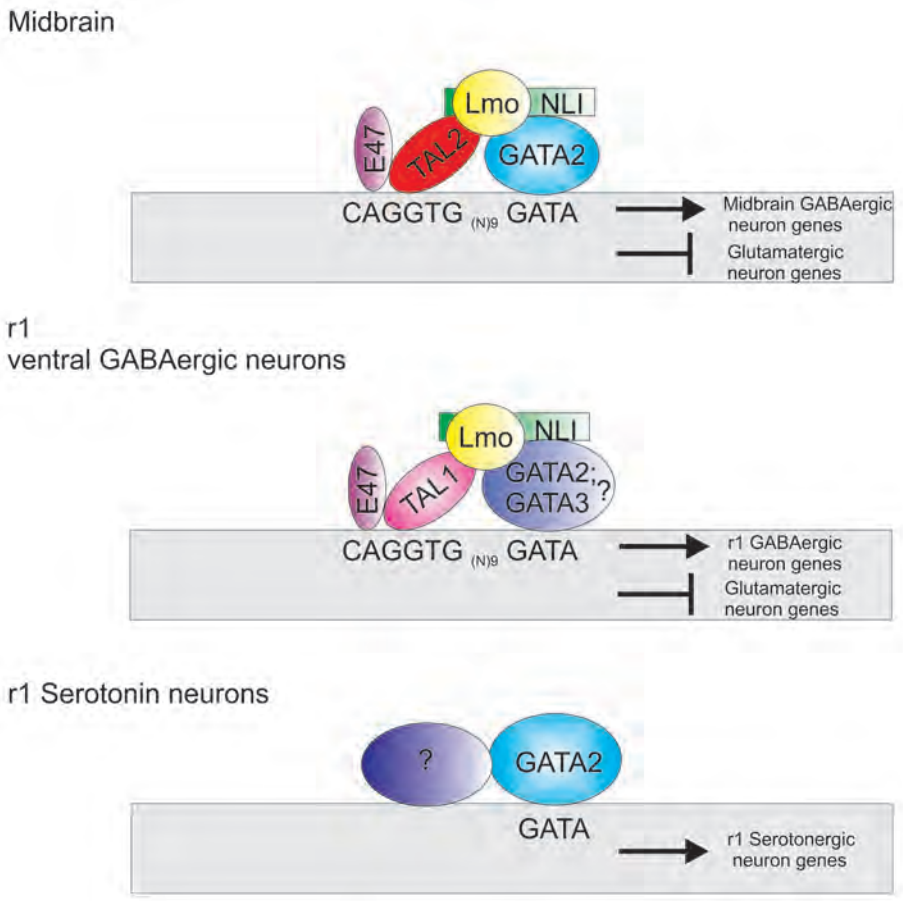


Figure 9. Proposed model of GATA-TAL transcription regulatory complexes mediating midbrain and rhombomere1 GABAergic neurogenesis. In the midbrain Tal2 and Gata2 are both required for complete activation of genes important for GABAergic neuron development. In the ventral rhombomere 1 (r1) Tal1 is specifically required for GABAergic neuron development. VTA-SNpr GABAergic neurons are specified in the r1 by Tal1 which potentially mediates r1 specific GABAergic neurogenesis together with Gata2 and Gata3.

CONCLUSIONS

Mature GABAergic neurons provide a functional balance to neuronal circuitries by coordinating and reducing the excitatory activity of other neurons. During embryonic development GABAergic neurotransmission can regulate several developmental steps from cell proliferation to migration. Disruption of GABA mediated inhibitory neurotransmission has been implicated lead to severe neurological diseases, including autism, epilepsy and schizophrenia. In addition, the effect of many sedative and addictive drugs is mediated by GABAergic neurotransmission.

Midbrain GABAergic neurons consist of several anatomical and functional subpopulations, which mediate important brain functions. Despite the functional relevance of midbrain GABAergic neurons to behavior of an individual, their molecular and developmental regulatory mechanisms have been rather poorly understood.

The aim of this study was to characterize the transcriptional regulatory mechanisms of GABAergic neurons during embryonic development in the diencephalon, midbrain and anterior hindbrain. We focused how transcription factors Gata2, Ascl1, Tal1 and Tal2 regulate GABAergic neuron differentiation in these brain regions.

We demonstrated the region specific functions of Ascl1 in the development of different diencephalon and midbrain GABAergic neurons. In these brain regions, Ascl1 regulated complex and even opposing aspects of neural progenitor proliferation and differentiation. We also characterized the requirement of Gata2 for GABAergic neurogenesis in diencephalon and revealed that *Gata2* performed post-mitotic selector gene function and was indispensable for the differentiation of distinct GABAergic neuron subtypes during diencephalon development.

Furthermore, we showed that Gata2 cofactors Tal1 and Tal2 are differentially regulating midbrain and anterior hindbrain GABAergic neuron differentiation and potentially analogous but molecularly distinct GATA/Tal transcriptional regulatory complexes operate the GABAergic differentiation in the midbrain and rhombomere1. These analyses demonstrated that there is genetic heterogeneity in the GABAergic differentiation mechanisms between different brain compartments as well as within them. Most importantly, this thesis work clarified the developmental origins of GABAergic neurons in different brain regions which were previously poorly characterized.

In the future, it will be particularly interesting to further characterize how different combinations of terminal selector genes of Gata and Tal families regulate differentiation of distinct neuronal populations during midbrain and hindbrain development.

Knowledge on the variation of developmental mechanisms generating diversity in different neuronal populations in the developing brain is the backbone for understanding brain functions and for the development of advanced therapies. Distinguishing in more detail the molecular interplay determinants of r1 derived VTA-SNpr GABAergic neurons would be especially intriguing. It is necessary to reveal the migratory pathways of these neurons, whether they utilize similar migratory mechanisms as in the forebrain or have developed unique way to relocate from r1 to the midbrain.

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REFERENCES

Akerman, C. J. and Cline, H. T. (2007). Refining the Roles of GABAergic Signaling during Neural Circuit Formation. *Trends Neurosci.* **30,** 382-389.
Anderson, S. A., Eisenstat, D. D., Shi, L. and Rubenstein, J. L. (1997). Interneuron Migration from Basal Forebrain to Neocortex: Dependence on Dlx Genes. *Science* **278,** 474-476.
Andrews, W., Barber, M., Hernadez-Miranda, L. R., Xian, J., Rakic, S., Sundaresan, V., Rabbitts, T. H., Pannell, R., Rabbitts, P., Thompson, H. et al. (2008). The Role of Slit-Robo Signaling in the Generation, Migration and Morphological Differentiation of Cortical Interneurons. *Dev. Biol.* **313,** 648-658.
Andrews, W. D., Barber, M. and Parnavelas, J. G. (2007). Slit-Robo Interactions during Cortical Development. *J. Anat.* **211,** 188-198.
Attardo, A., Calegari, F., Haubensak, W., Wilsch-Brauninger, M. and Huttner, W. B. (2008). Live Imaging at the Onset of Cortical Neurogenesis Reveals Differential Appearance of the Neuronal Phenotype in Apical Versus Basal Progenitor Progeny. *PLoS One* **3,** e2388.
Baek, J. H., Hatakeyama, J., Sakamoto, S., Ohtsuka, T. and Kageyama, R. (2006). Persistent and High Levels of Hes1 Expression Regulate Boundary Formation in the Developing Central Nervous System. *Development* **133,** 2467-2476.
Balaskas, N., Ribeiro, A., Panovska, J., Dessaud, E., Sasai, N., Page, K. M., Briscoe, J. and Ribes, V. (2012). Gene Regulatory Logic for Reading the Sonic Hedgehog Signaling Gradient in the Vertebrate Neural Tube. *Cell* **148,** 273-284.
Behar, T. N., Li, Y. X., Tran, H. T., Ma, W., Dunlap, V., Scott, C. and Barker, J. L. (1996). GABA Stimulates Chemotaxis and Chemokinesis of Embryonic Cortical Neurons Via Calcium-Dependent Mechanisms. *J. Neurosci.* **16,** 1808-1818.
Behar, T. N., Schaffner, A. E., Scott, C. A., Greene, C. L. and Barker, J. L. (2000). GABA Receptor Antagonists Modulate Postmitotic Cell Migration in Slice Cultures of Embryonic Rat Cortex. *Cereb. Cortex* **10,** 899-909.
Ben-Ari, Y., Gaiarsa, J. L., Tyzio, R. and Khazipov, R. (2007). GABA: A Pioneer Transmitter that Excites Immature Neurons and Generates Primitive Oscillations. *Physiol. Rev.* **87,** 1215-1284.
Bergsland, M., Ramskold, D., Zaouter, C., Klum, S., Sandberg, R. and Muhr, J. (2011). Sequentially Acting Sox Transcription Factors in Neural Lineage Development. *Genes Dev.* **25,** 2453-2464.
Bertrand, N., Castro, D. S. and Guillemot, F. (2002). Proneural Genes and the Specification of Neural Cell Types. *Nat. Rev. Neurosci.* **3,** 517-530.
Bjorklund, A. and Dunnett, S. B. (2007). Dopamine Neuron Systems in the Brain: An Update. *Trends Neurosci.* **30,** 194-202.
Bormann, J. (2000). The 'ABC' of GABA Receptors. *Trends Pharmacol. Sci.* **21,** 16-19.
Bortone, D. and Polleux, F. (2009). KCC2 Expression Promotes the Termination of Cortical Interneuron Migration in a Voltage-Sensitive Calcium-Dependent Manner. *Neuron* **62,** 53-71.
Bradley, C. K., Takano, E. A., Hall, M. A., Gothert, J. R., Harvey, A. R., Begley, C. G. and van Eekelen, J. A. (2006). The Essential Haematopoietic Transcription Factor Scl is also Critical for Neuronal Development. *Eur. J. Neurosci.* **23,** 1677-1689.
Bresnick, E. H., Martowicz, M. L., Pal, S. and Johnson, K. D. (2005). Developmental Control Via GATA Factor Interplay at Chromatin Domains. *J. Cell. Physiol.* **205,** 1-9.
Briscoe, J. and Ericson, J. (1999). The Specification of Neuronal Identity by Graded Sonic Hedgehog Signalling. *Semin. Cell Dev. Biol.* **10,** 353-362.

Briscoe, J., Pierani, A., Jessell, T. M. and Ericson, J. (2000). A Homeodomain Protein Code Specifies Progenitor Cell Identity and Neuronal Fate in the Ventral Neural Tube. *Cell* **101,** 435-445.
Broccoli, V., Boncinelli, E. and Wurst, W. (1999). The Caudal Limit of Otx2 Expression Positions the Isthmic Organizer. *Nature* **401,** 164-168.
Bromberg-Martin, E. S., Matsumoto, M. and Hikosaka, O. (2010). Dopamine in Motivational Control: Rewarding, Aversive, and Alerting. *Neuron* **68,** 815-834.
Brown, L., Cheng, J. T., Chen, Q., Siciliano, M. J., Crist, W., Buchanan, G. and Baer, R. (1990). Site-Specific Recombination of the Tal-1 Gene is a Common Occurrence in Human T Cell Leukemia. *EMBO J.* **9,** 3343-3351.
Bucher, K., Sofroniew, M. V., Pannell, R., Impey, H., Smith, A. J., Torres, E. M., Dunnett, S. B., Jin, Y., Baer, R. and Rabbitts, T. H. (2000). The T Cell Oncogene Tal2 is Necessary for Normal Development of the Mouse Brain. *Dev. Biol.* **227,** 533-544.
Bulfone, A., Wang, F., Hevner, R., Anderson, S., Cutforth, T., Chen, S., Meneses, J., Pedersen, R., Axel, R. and Rubenstein, J. L. (1998). An Olfactory Sensory Map Develops in the Absence of Normal Projection Neurons Or GABAergic Interneurons. *Neuron* **21,** 1273-1282.
Bylund, M., Andersson, E., Novitch, B. G. and Muhr, J. (2003). Vertebrate Neurogenesis is Counteracted by Sox1-3 Activity. *Nat. Neurosci.* **6,** 1162-1168.
Campbell, K. (2003). Dorsal-Ventral Patterning in the Mammalian Telencephalon. *Curr. Opin. Neurobiol.* **13,** 50-56.
Cantor, A. B. and Orkin, S. H. (2002). Transcriptional Regulation of Erythropoiesis: An Affair Involving Multiple Partners. *Oncogene* **21,** 3368-3376.
Carletti, B. and Rossi, F. (2008). Neurogenesis in the Cerebellum. *Neuroscientist* **14,** 91-100.
Casarosa, S., Fode, C. and Guillemot, F. (1999). Mash1 Regulates Neurogenesis in the Ventral Telencephalon. *Development* **126,** 525-534.
Castro, D. S. and Guillemot, F. (2011). Old and New Functions of Proneural Factors Revealed by the Genome-Wide Characterization of their Transcriptional Targets. *Cell. Cycle* **10,**.
Castro, D. S., Martynoga, B., Parras, C., Ramesh, V., Pacary, E., Johnston, C., Drechsel, D., Lebel-Potter, M., Garcia, L. G., Hunt, C. et al. (2011). A Novel Function of the Proneural Factor Ascl1 in Progenitor Proliferation Identified by Genome-Wide Characterization of its Targets. *Genes Dev.* **25,** 930-945.
Chen, H., Thiagalingam, A., Chopra, H., Borges, M. W., Feder, J. N., Nelkin, B. D., Baylin, S. B. and Ball, D. W. (1997). Conservation of the Drosophila Lateral Inhibition Pathway in Human Lung Cancer: A Hairy-Related Protein (HES-1) Directly Represses Achaete-Scute Homolog-1 Expression. *Proc. Natl. Acad. Sci. U. S. A.* **94,** 5355-5360.
Chen, L., Guo, Q. and Li, J. Y. (2009). Transcription Factor Gbx2 Acts Cell-Nonautonomously to Regulate the Formation of Lineage-Restriction Boundaries of the Thalamus. *Development* **136,** 1317-1326.
Chen, Q., Cheng, J. T., Tasi, L. H., Schneider, N., Buchanan, G., Carroll, A., Crist, W., Ozanne, B., Siciliano, M. J. and Baer, R. (1990). The Tal Gene Undergoes Chromosome Translocation in T Cell Leukemia and Potentially Encodes a Helix-Loop-Helix Protein. *EMBO J.* **9,** 415-424.
Cheng, L., Arata, A., Mizuguchi, R., Qian, Y., Karunaratne, A., Gray, P. A., Arata, S., Shirasawa, S., Bouchard, M., Luo, P. et al. (2004). Tlx3 and Tlx1 are Post-Mitotic Selector Genes Determining Glutamatergic Over GABAergic Cell Fates. *Nat. Neurosci.* **7,** 510-517.
Cobos, I., Calcagnotto, M. E., Vilaythong, A. J., Thwin, M. T., Noebels, J. L., Baraban, S. C. and Rubenstein, J. L. (2005). Mice Lacking Dlx1 show Subtype-Specific Loss of Interneurons, Reduced Inhibition and Epilepsy. *Nat. Neurosci.* **8,** 1059-1068.

Cohen, J. Y., Haesler, S., Vong, L., Lowell, B. B. and Uchida, N. (2012). Neuron-Type-Specific Signals for Reward and Punishment in the Ventral Tegmental Area. *Nature* **482,** 85-88.
Crandall, J. E., McCarthy, D. M., Araki, K. Y., Sims, J. R., Ren, J. Q. and Bhide, P. G. (2007). Dopamine Receptor Activation Modulates GABA Neuron Migration from the Basal Forebrain to the Cerebral Cortex. *J. Neurosci.* **27,** 3813-3822.
Craven, S. E., Lim, K. C., Ye, W., Engel, J. D., de Sauvage, F. and Rosenthal, A. (2004). Gata2 Specifies Serotonergic Neurons Downstream of Sonic Hedgehog. *Development* **131,** 1165-1173.
Crossley, P. H., Martinez, S. and Martin, G. R. (1996). Midbrain Development Induced by FGF8 in the Chick Embryo. *Nature* **380,** 66-68.
Cuzon, V. C., Yeh, P. W., Cheng, Q. and Yeh, H. H. (2006). Ambient GABA Promotes Cortical Entry of Tangentially Migrating Cells Derived from the Medial Ganglionic Eminence. *Cereb. Cortex* **16,** 1377-1388.
Danielian, P. S., Muccino, D., Rowitch, D. H., Michael, S. K. and McMahon, A. P. (1998). Modification of Gene Activity in Mouse Embryos in Utero by a Tamoxifen-Inducible Form of Cre Recombinase. *Curr. Biol.* **8,** 1323-1326.
Del Barrio, M. G., Taveira-Marques, R., Muroyama, Y., Yuk, D. I., Li, S., Wines-Samuelson, M., Shen, J., Smith, H. K., Xiang, M., Rowitch, D. et al. (2007). A Regulatory Network Involving Foxn4, Mash1 and Delta-Like 4/Notch1 Generates V2a and V2b Spinal Interneurons from a Common Progenitor Pool. *Development* **134,** 3427-3436.
Del Bene, F., Wehman, A. M., Link, B. A. and Baier, H. (2008). Regulation of Neurogenesis by Interkinetic Nuclear Migration through an Apical-Basal Notch Gradient. *Cell* **134,** 1055-1065.
Dessaud, E., McMahon, A. P. and Briscoe, J. (2008). Pattern Formation in the Vertebrate Neural Tube: A Sonic Hedgehog Morphogen-Regulated Transcriptional Network. *Development* **135,** 2489-2503.
Echevarria, D., Vieira, C., Gimeno, L. and Martinez, S. (2003). Neuroepithelial Secondary Organizers and Cell Fate Specification in the Developing Brain. *Brain Res. Brain Res. Rev.* **43,** 179-191.
Englund, C., Fink, A., Lau, C., Pham, D., Daza, R. A., Bulfone, A., Kowalczyk, T. and Hevner, R. F. (2005). Pax6, Tbr2, and Tbr1 are Expressed Sequentially by Radial Glia, Intermediate Progenitor Cells, and Postmitotic Neurons in Developing Neocortex. *J. Neurosci.* **25,** 247-251.
Episkopou, V. (2005). SOX2 Functions in Adult Neural Stem Cells. *Trends Neurosci.* **28,** 219-221.
Ericson, J., Muhr, J., Placzek, M., Lints, T., Jessell, T. M. and Edlund, T. (1995). Sonic Hedgehog Induces the Differentiation of Ventral Forebrain Neurons: A Common Signal for Ventral Patterning within the Neural Tube. *Cell* **81,** 747-756.
Ericson, J., Rashbass, P., Schedl, A., Brenner-Morton, S., Kawakami, A., van Heyningen, V., Jessell, T. M. and Briscoe, J. (1997). Pax6 Controls Progenitor Cell Identity and Neuronal Fate in Response to Graded Shh Signaling. *Cell* **90,** 169-180.
Farah, M. H., Olson, J. M., Sucic, H. B., Hume, R. I., Tapscott, S. J. and Turner, D. L. (2000). Generation of Neurons by Transient Expression of Neural bHLH Proteins in Mammalian Cells. *Development* **127,** 693-702.
Ferri, A. L., Cavallaro, M., Braida, D., Di Cristofano, A., Canta, A., Vezzani, A., Ottolenghi, S., Pandolfi, P. P., Sala, M., DeBiasi, S. et al. (2004). Sox2 Deficiency Causes Neurodegeneration and Impaired Neurogenesis in the Adult Mouse Brain. *Development* **131,** 3805-3819.

Fields, H. L., Hjelmstad, G. O., Margolis, E. B. and Nicola, S. M. (2007). Ventral Tegmental Area Neurons in Learned Appetitive Behavior and Positive Reinforcement. *Annu. Rev. Neurosci.* **30,** 289-316.

Fietz, S. A. and Huttner, W. B. (2011). Cortical Progenitor Expansion, Self-Renewal and Neurogenesis-a Polarized Perspective. *Curr. Opin. Neurobiol.* **21,** 23-35.

Fietz, S. A., Kelava, I., Vogt, J., Wilsch-Brauninger, M., Stenzel, D., Fish, J. L., Corbeil, D., Riehn, A., Distler, W., Nitsch, R. et al. (2010). OSVZ Progenitors of Human and Ferret Neocortex are Epithelial-Like and Expand by Integrin Signaling. *Nat. Neurosci.* **13,** 690-699.

Fode, C., Ma, Q., Casarosa, S., Ang, S. L., Anderson, D. J. and Guillemot, F. (2000). A Role for Neural Determination Genes in Specifying the Dorsoventral Identity of Telencephalic Neurons. *Genes Dev.* **14,** 67-80.

Funahashi, J., Okafuji, T., Ohuchi, H., Noji, S., Tanaka, H. and Nakamura, H. (1999). Role of Pax-5 in the Regulation of a Mid-Hindbrain Organizer's Activity. *Dev. Growth Differ.* **41,** 59-72.

Garcia-Lopez, R., Vieira, C., Echevarria, D. and Martinez, S. (2004). Fate Map of the Diencephalon and the Zona Limitans at the 10-Somites Stage in Chick Embryos. *Dev. Biol.* **268,** 514-530.

Ge, S., Pradhan, D. A., Ming, G. L. and Song, H. (2007). GABA Sets the Tempo for Activity-Dependent Adult Neurogenesis. *Trends Neurosci.* **30,** 1-8.

Ge, W., He, F., Kim, K. J., Blanchi, B., Coskun, V., Nguyen, L., Wu, X., Zhao, J., Heng, J. I., Martinowich, K. et al. (2006). Coupling of Cell Migration with Neurogenesis by Proneural bHLH Factors. *Proc. Natl. Acad. Sci. U. S. A.* **103,** 1319-1324.

Glasgow, S. M., Henke, R. M., Macdonald, R. J., Wright, C. V. and Johnson, J. E. (2005). Ptf1a Determines GABAergic Over Glutamatergic Neuronal Cell Fate in the Spinal Cord Dorsal Horn. *Development* **132,** 5461-5469.

Goto, T., Mitsuhashi, T. and Takahashi, T. (2004). Altered Patterns of Neuron Production in the p27 Knockout Mouse. *Dev. Neurosci.* **26,** 208-217.

Gotz, M. and Huttner, W. B. (2005). The Cell Biology of Neurogenesis. *Nat. Rev. Mol. Cell Biol.* **6,** 777-788.

Gowan, K., Helms, A. W., Hunsaker, T. L., Collisson, T., Ebert, P. J., Odom, R. and Johnson, J. E. (2001). Crossinhibitory Activities of Ngn1 and Math1 Allow Specification of Distinct Dorsal Interneurons. *Neuron* **31,** 219-232.

Graham, V., Khudyakov, J., Ellis, P. and Pevny, L. (2003). SOX2 Functions to Maintain Neural Progenitor Identity. *Neuron* **39,** 749-765.

Gray, P. A. (2008). Transcription Factors and the Genetic Organization of Brain Stem Respiratory Neurons. *J. Appl. Physiol.* **104,** 1513-1521.

Green, A. R. and Begley, C. G. (1992). SCL and Related Hemopoietic Helix-Loop-Helix Transcription Factors. *Int. J. Cell Cloning* **10,** 269-276.

Gross, M. K., Dottori, M. and Goulding, M. (2002). Lbx1 Specifies Somatosensory Association Interneurons in the Dorsal Spinal Cord. *Neuron* **34,** 535-549.

Grossmann, K. S., Giraudin, A., Britz, O., Zhang, J. and Goulding, M. (2010). Genetic Dissection of Rhythmic Motor Networks in Mice. *Prog. Brain Res.* **187,** 19-37.

Guillemot, F. (2007). Spatial and Temporal Specification of Neural Fates by Transcription Factor Codes. *Development* **134,** 3771-3780.

Guillemot, F., Lo, L. C., Johnson, J. E., Auerbach, A., Anderson, D. J. and Joyner, A. L. (1993). Mammalian Achaete-Scute Homolog 1 is Required for the Early Development of Olfactory and Autonomic Neurons. *Cell* **75,** 463-476.

Guimera, J., Weisenhorn, D. V. and Wurst, W. (2006). Megane/Heslike is Required for Normal GABAergic Differentiation in the Mouse Superior Colliculus. *Development* **133,** 3847-3857.

Hall, M. A., Curtis, D. J., Metcalf, D., Elefanty, A. G., Sourris, K., Robb, L., Gothert, J. R., Jane, S. M. and Begley, C. G. (2003). The Critical Regulator of Embryonic Hematopoiesis, SCL, is Vital in the Adult for Megakaryopoiesis, Erythropoiesis, and Lineage Choice in CFU-S12. *Proc. Natl. Acad. Sci. U. S. A.* **100,** 992-997.

Harfe, B. D., Scherz, P. J., Nissim, S., Tian, H., McMahon, A. P. and Tabin, C. J. (2004). Evidence for an Expansion-Based Temporal Shh Gradient in Specifying Vertebrate Digit Identities. *Cell* **118,** 517-528.

Harrington, M. E. (1997). The Ventral Lateral Geniculate Nucleus and the Intergeniculate Leaflet: Interrelated Structures in the Visual and Circadian Systems. *Neurosci. Biobehav. Rev.* **21,** 705-727.

Hashimoto-Torii, K., Motoyama, J., Hui, C. C., Kuroiwa, A., Nakafuku, M. and Shimamura, K. (2003). Differential Activities of Sonic Hedgehog Mediated by Gli Transcription Factors Define Distinct Neuronal Subtypes in the Dorsal Thalamus. *Mech. Dev.* **120,** 1097-1111.

Hatakeyama, J., Bessho, Y., Katoh, K., Ookawara, S., Fujioka, M., Guillemot, F. and Kageyama, R. (2004). Hes Genes Regulate Size, Shape and Histogenesis of the Nervous System by Control of the Timing of Neural Stem Cell Differentiation. *Development* **131,** 5539-5550.

Haubensak, W., Attardo, A., Denk, W. and Huttner, W. B. (2004). Neurons Arise in the Basal Neuroepithelium of the Early Mammalian Telencephalon: A Major Site of Neurogenesis. *Proc. Natl. Acad. Sci. U. S. A.* **101,** 3196-3201.

Haugas, M., Lillevali, K., Hakanen, J. and Salminen, M. (2010). Gata2 is Required for the Development of Inner Ear Semicircular Ducts and the Surrounding Perilymphatic Space. *Dev. Dyn.* **239,** 2452-2469.

Hebert, J. M. and McConnell, S. K. (2000). Targeting of Cre to the Foxg1 (BF-1) Locus Mediates loxP Recombination in the Telencephalon and Other Developing Head Structures. *Dev. Biol.* **222,** 296-306.

Hobert, O. (2005). Specification of the Nervous System. *WormBook*, 1-19.

Hobert, O. (2008). Regulatory Logic of Neuronal Diversity: Terminal Selector Genes and Selector Motifs. *Proc. Natl. Acad. Sci. U. S. A.* **105,** 20067-20071.

Hobert, O. (2011). Regulation of Terminal Differentiation Programs in the Nervous System. *Annu. Rev. Cell Dev. Biol.* **27,** 681-696.

Hobert, O., Carrera, I. and Stefanakis, N. (2010). The Molecular and Gene Regulatory Signature of a Neuron. *Trends Neurosci.* **33,** 435-445.

Hong, S., Jhou, T. C., Smith, M., Saleem, K. S. and Hikosaka, O. (2011). Negative Reward Signals from the Lateral Habenula to Dopamine Neurons are Mediated by Rostromedial Tegmental Nucleus in Primates. *J. Neurosci.* **31,** 11457-11471.

Hoshino, M., Nakamura, S., Mori, K., Kawauchi, T., Terao, M., Nishimura, Y. V., Fukuda, A., Fuse, T., Matsuo, N., Sone, M. et al. (2005). Ptf1a, a bHLH Transcriptional Gene, Defines GABAergic Neuronal Fates in Cerebellum. *Neuron* **47,** 201-213.

Houart, C., Caneparo, L., Heisenberg, C., Barth, K., Take-Uchi, M. and Wilson, S. (2002). Establishment of the Telencephalon during Gastrulation by Local Antagonism of Wnt Signaling. *Neuron* **35,** 255-265.

Hsu, H. L., Huang, L., Tsan, J. T., Funk, W., Wright, W. E., Hu, J. S., Kingston, R. E. and Baer, R. (1994). Preferred Sequences for DNA Recognition by the TAL1 Helix-Loop-Helix Proteins. *Mol. Cell. Biol.* **14,** 1256-1265.

Huang, Z. J. (2009). Activity-Dependent Development of Inhibitory Synapses and Innervation Pattern: Role of GABA Signalling and Beyond. *J. Physiol.* **587,** 1881-1888.
Irving, C. and Mason, I. (2000). Signalling by FGF8 from the Isthmus Patterns Anterior Hindbrain and Establishes the Anterior Limit of Hox Gene Expression. *Development* **127,** 177-186.
Jacobs, F. M., van Erp, S., van der Linden, A. J., von Oerthel, L., Burbach, J. P. and Smidt, M. P. (2009). Pitx3 Potentiates Nurr1 in Dopamine Neuron Terminal Differentiation through Release of SMRT-Mediated Repression. *Development* **136,** 531-540.
Jhou, T. C., Fields, H. L., Baxter, M. G., Saper, C. B. and Holland, P. C. (2009a). The Rostromedial Tegmental Nucleus (RMTg), a GABAergic Afferent to Midbrain Dopamine Neurons, Encodes Aversive Stimuli and Inhibits Motor Responses. *Neuron* **61,** 786-800.
Jhou, T. C., Geisler, S., Marinelli, M., Degarmo, B. A. and Zahm, D. S. (2009b). The Mesopontine Rostromedial Tegmental Nucleus: A Structure Targeted by the Lateral Habenula that Projects to the Ventral Tegmental Area of Tsai and Substantia Nigra Compacta. *J. Comp. Neurol.* **513,** 566-596.
Johnson, J. E., Birren, S. J., Saito, T. and Anderson, D. J. (1992). DNA Binding and Transcriptional Regulatory Activity of Mammalian Achaete-Scute Homologous (MASH) Proteins Revealed by Interaction with a Muscle-Specific Enhancer. *Proc. Natl. Acad. Sci. U. S. A.* **89,** 3596-3600.
Joshi, K., Lee, S., Lee, B., Lee, J. W. and Lee, S. K. (2009). LMO4 Controls the Balance between Excitatory and Inhibitory Spinal V2 Interneurons. *Neuron* **61,** 839-851.
Jovanovic, J. N. and Thomson, A. M. (2011). Development of Cortical GABAergic Innervation. *Front. Cell. Neurosci.* **5,** 14.
Joyner, A. L. (1996). Engrailed, Wnt and Pax Genes Regulate Midbrain--Hindbrain Development. *Trends Genet.* **12,** 15-20.
Joyner, A. L., Liu, A. and Millet, S. (2000). Otx2, Gbx2 and Fgf8 Interact to Position and Maintain a Mid-Hindbrain Organizer. *Curr. Opin. Cell Biol.* **12,** 736-741.
Joyner, A. L. and Zervas, M. (2006). Genetic Inducible Fate Mapping in Mouse: Establishing Genetic Lineages and Defining Genetic Neuroanatomy in the Nervous System. *Dev. Dyn.* **235,** 2376-2385.
Kageyama, R., Niwa, Y. and Shimojo, H. (2009). Rhythmic Gene Expression in Somite Formation and Neural Development. *Mol. Cells* **27,** 497-502.
Kageyama, R., Ohtsuka, T., Hatakeyama, J. and Ohsawa, R. (2005). Roles of bHLH Genes in Neural Stem Cell Differentiation. *Exp. Cell Res.* **306,** 343-348.
Kageyama, R., Ohtsuka, T., Ohsawa, R. and Hatakeyama, J. (2009). Helix-Loop-Helix (bHLH) Proteins: Hes Family. In *Developmental Neurobiology* (ed. G. Lemke), pp. 156-164. Academic Print: Elsevier.
Kageyama, R., Ohtsuka, T., Shimojo, H. and Imayoshi, I. (2008). Dynamic Notch Signaling in Neural Progenitor Cells and a Revised View of Lateral Inhibition. *Nat. Neurosci.* **11,** 1247-1251.
Kageyama, R., Ohtsuka, T., Shimojo, H. and Imayoshi, I. (2009). Dynamic Regulation of Notch Signaling in Neural Progenitor Cells. *Curr. Opin. Cell Biol.* **21,** 733-740.
Kala, K., Haugas, M., Lillevali, K., Guimera, J., Wurst, W., Salminen, M. and Partanen, J. (2009). Gata2 is a Tissue-Specific Post-Mitotic Selector Gene for Midbrain GABAergic Neurons. *Development* **136,** 253-262.
Kala, K., Jukkola, T., Pata, I. and Partanen, J. (2008). Analysis of the Midbrain-Hindbrain Boundary Cell Fate using a Boundary Cell-Specific Cre-Mouse Strain. *Genesis* **46,** 29-36.
Karunaratne, A., Hargrave, M., Poh, A. and Yamada, T. (2002). GATA Proteins Identify a Novel Ventral Interneuron Subclass in the Developing Chick Spinal Cord. *Dev. Biol.* **249,** 30-43.

Kataoka, A. and Shimogori, T. (2008). Fgf8 Controls Regional Identity in the Developing Thalamus. *Development* **135,** 2873-2881.
Kawaguchi, Y. and Kubota, Y. (1997). GABAergic Cell Subtypes and their Synaptic Connections in Rat Frontal Cortex. *Cereb. Cortex* **7,** 476-486.
Kiecker, C. and Lumsden, A. (2004). Hedgehog Signaling from the ZLI Regulates Diencephalic Regional Identity. *Nat. Neurosci.* **7,** 1242-1249.
Kimmel, R. A., Turnbull, D. H., Blanquet, V., Wurst, W., Loomis, C. A. and Joyner, A. L. (2000). Two Lineage Boundaries Coordinate Vertebrate Apical Ectodermal Ridge Formation. *Genes Dev.* **14,** 1377-1389.
Kitamura, K., Miura, H., Yanazawa, M., Miyashita, T. and Kato, K. (1997). Expression Patterns of Brx1 (Rieg Gene), Sonic Hedgehog, Nkx2.2, Dlx1 and Arx during Zona Limitans Intrathalamica and Embryonic Ventral Lateral Geniculate Nuclear Formation. *Mech. Dev.* **67,** 83-96.
Kriegstein, A., Noctor, S. and Martinez-Cerdeno, V. (2006). Patterns of Neural Stem and Progenitor Cell Division may Underlie Evolutionary Cortical Expansion. *Nat. Rev. Neurosci.* **7,** 883-890.
Kurek, D., Garinis, G. A., van Doorninck, J. H., van der Wees, J. and Grosveld, F. G. (2007). Transcriptome and Phenotypic Analysis Reveals Gata3-Dependent Signalling Pathways in Murine Hair Follicles. *Development* **134,** 261-272.
Lagutin, O. V., Zhu, C. C., Kobayashi, D., Topczewski, J., Shimamura, K., Puelles, L., Russell, H. R., McKinnon, P. J., Solnica-Krezel, L. and Oliver, G. (2003). Six3 Repression of Wnt Signaling in the Anterior Neuroectoderm is Essential for Vertebrate Forebrain Development. *Genes Dev.* **17,** 368-379.
Lahti, L., Peltopuro, P., Piepponen, T. P. and Partanen, J. (2012). Cell-Autonomous FGF Signaling Regulates Anteroposterior Patterning and Neuronal Differentiation in the Mesodiencephalic Dopaminergic Progenitor Domain. *Development* **139,** 894-905.
Lahti, L., Saarimaki-Vire, J., Rita, H. and Partanen, J. (2011). FGF Signaling Gradient Maintains Symmetrical Proliferative Divisions of Midbrain Neuronal Progenitors. *Dev. Biol.* **349,** 270-282.
Lange, C., Huttner, W. B. and Calegari, F. (2009). Cdk4/cyclinD1 Overexpression in Neural Stem Cells Shortens G1, Delays Neurogenesis, and Promotes the Generation and Expansion of Basal Progenitors. *Cell. Stem Cell.* **5,** 320-331.
Lavado, A., Lagutin, O. V. and Oliver, G. (2008). Six3 Inactivation Causes Progressive Caudalization and Aberrant Patterning of the Mammalian Diencephalon. *Development* **135,** 441-450.
Lee, K. J. and Jessell, T. M. (1999). The Specification of Dorsal Cell Fates in the Vertebrate Central Nervous System. *Annu. Rev. Neurosci.* **22,** 261-294.
Lee, S. K. and Pfaff, S. L. (2003). Synchronization of Neurogenesis and Motor Neuron Specification by Direct Coupling of bHLH and Homeodomain Transcription Factors. *Neuron* **38,** 731-745.
Li, H., Tornberg, J., Kaila, K., Airaksinen, M. S. and Rivera, C. (2002). Patterns of Cation-Chloride Cotransporter Expression during Embryonic Rodent CNS Development. *Eur. J. Neurosci.* **16,** 2358-2370.
Li, S., Misra, K., Matise, M. P. and Xiang, M. (2005). Foxn4 Acts Synergistically with Mash1 to Specify Subtype Identity of V2 Interneurons in the Spinal Cord. *Proc. Natl. Acad. Sci. U. S. A.* **102,** 10688-10693.
Lillien, L. and Raphael, H. (2000). BMP and FGF Regulate the Development of EGF-Responsive Neural Progenitor Cells. *Development* **127,** 4993-5005.
Liu, A. and Joyner, A. L. (2001). Early anterior/posterior Patterning of the Midbrain and Cerebellum. *Annu. Rev. Neurosci.* **24,** 869-896.

Liu, C., Maejima, T., Wyler, S. C., Casadesus, G., Herlitze, S. and Deneris, E. S. (2010). Pet-1 is Required Across Different Stages of Life to Regulate Serotonergic Function. *Nat. Neurosci.* **13,** 1190-1198.

Liu, X., Wang, Q., Haydar, T. F. and Bordey, A. (2005). Nonsynaptic GABA Signaling in Postnatal Subventricular Zone Controls Proliferation of GFAP-Expressing Progenitors. *Nat. Neurosci.* **8,** 1179-1187.

Lopez-Bendito, G., Sanchez-Alcaniz, J. A., Pla, R., Borrell, V., Pico, E., Valdeolmillos, M. and Marin, O. (2008). Chemokine Signaling Controls Intracortical Migration and Final Distribution of GABAergic Interneurons. *J. Neurosci.* **28,** 1613-1624.

LoTurco, J. J., Owens, D. F., Heath, M. J., Davis, M. B. and Kriegstein, A. R. (1995). GABA and Glutamate Depolarize Cortical Progenitor Cells and Inhibit DNA Synthesis. *Neuron* **15,** 1287-1298.

Lukaszewicz, A., Savatier, P., Cortay, V., Kennedy, H. and Dehay, C. (2002). Contrasting Effects of Basic Fibroblast Growth Factor and Neurotrophin 3 on Cell Cycle Kinetics of Mouse Cortical Stem Cells. *J. Neurosci.* **22,** 6610-6622.

Maric, D., Fiorio Pla, A., Chang, Y. H. and Barker, J. L. (2007). Self-Renewing and Differentiating Properties of Cortical Neural Stem Cells are Selectively Regulated by Basic Fibroblast Growth Factor (FGF) Signaling Via Specific FGF Receptors. *J. Neurosci.* **27,** 1836-1852.

Marin, O. and Rubenstein, J. L. (2001). A Long, Remarkable Journey: Tangential Migration in the Telencephalon. *Nat. Rev. Neurosci.* **2,** 780-790.

Marin, O. and Rubenstein, J. L. (2003). Cell Migration in the Forebrain. *Annu. Rev. Neurosci.* **26,** 441-483.

Martinez, S. (2001). The Isthmic Organizer and Brain Regionalization. *Int. J. Dev. Biol.* **45,** 367-371.

Martinez, S., Crossley, P. H., Cobos, I., Rubenstein, J. L. and Martin, G. R. (1999). FGF8 Induces Formation of an Ectopic Isthmic Organizer and Isthmocerebellar Development Via a Repressive Effect on Otx2 Expression. *Development* **126,** 1189-1200.

Mason, I., Chambers, D., Shamim, H., Walshe, J. and Irving, C. (2000). Regulation and Function of FGF8 in Patterning of Midbrain and Anterior Hindbrain. *Biochem. Cell Biol.* **78,** 577-584.

Massari, M. E. and Murre, C. (2000). Helix-Loop-Helix Proteins: Regulators of Transcription in Eucaryotic Organisms. *Mol. Cell. Biol.* **20,** 429-440.

Matsumoto, K., Nishihara, S., Kamimura, M., Shiraishi, T., Otoguro, T., Uehara, M., Maeda, Y., Ogura, K., Lumsden, A. and Ogura, T. (2004). The Prepattern Transcription Factor Irx2, a Target of the FGF8/MAP Kinase Cascade, is Involved in Cerebellum Formation. *Nat. Neurosci.* **7,** 605-612.

Matsumoto, M. and Hikosaka, O. (2007). Lateral Habenula as a Source of Negative Reward Signals in Dopamine Neurons. *Nature* **447,** 1111-1115.

Matsunaga, E., Araki, I. and Nakamura, H. (2000). Pax6 Defines the Di-Mesencephalic Boundary by Repressing En1 and Pax2. *Development* **127,** 2357-2365.

Millet, S., Campbell, K., Epstein, D. J., Losos, K., Harris, E. and Joyner, A. L. (1999). A Role for Gbx2 in Repression of Otx2 and Positioning the mid/hindbrain Organizer. *Nature* **401,** 161-164.

Miyoshi, G., Bessho, Y., Yamada, S. and Kageyama, R. (2004). Identification of a Novel Basic Helix-Loop-Helix Gene, Heslike, and its Role in GABAergic Neurogenesis. *J. Neurosci.* **24,** 3672-3682.

Mizuguchi, R., Kriks, S., Cordes, R., Gossler, A., Ma, Q. and Goulding, M. (2006). Ascl1 and Gsh1/2 Control Inhibitory and Excitatory Cell Fate in Spinal Sensory Interneurons. *Nat. Neurosci.* **9,** 770-778.

Molkentin, J. D. (2000). The Zinc Finger-Containing Transcription Factors GATA-4, -5, and -6. Ubiquitously Expressed Regulators of Tissue-Specific Gene Expression. *J. Biol. Chem.* **275,** 38949-38952.
Mori, S., Sugawara, S., Kikuchi, T., Tanji, M., Narumi, O., Stoykova, A., Nishikawa, S. I. and Yokota, Y. (1999). The Leukemic Oncogene Tal-2 is Expressed in the Developing Mouse Brain. *Brain Res. Mol. Brain Res.* **64,** 199-210.
Nakajima, K. (2007). Control of tangential/non-Radial Migration of Neurons in the Developing Cerebral Cortex. *Neurochem. Int.* **51,** 121-131.
Nakamura, H., Katahira, T., Matsunaga, E. and Sato, T. (2005). Isthmus Organizer for Midbrain and Hindbrain Development. *Brain Res. Brain Res. Rev.* **49,** 120-126.
Nakamura, H., Sato, T. and Suzuki-Hirano, A. (2008). Isthmus Organizer for Mesencephalon and Metencephalon. *Dev. Growth Differ.* **50 Suppl 1,** S113-8.
Nakatani, T., Minaki, Y., Kumai, M. and Ono, Y. (2007). Helt Determines GABAergic Over Glutamatergic Neuronal Fate by Repressing Ngn Genes in the Developing Mesencephalon. *Development* **134,** 2783-2793.
Nardelli, J., Thiesson, D., Fujiwara, Y., Tsai, F. Y. and Orkin, S. H. (1999). Expression and Genetic Interaction of Transcription Factors GATA-2 and GATA-3 during Development of the Mouse Central Nervous System. *Dev. Biol.* **210,** 305-321.
Nguyen, L., Besson, A., Heng, J. I., Schuurmans, C., Teboul, L., Parras, C., Philpott, A., Roberts, J. M. and Guillemot, F. (2006). P27kip1 Independently Promotes Neuronal Differentiation and Migration in the Cerebral Cortex. *Genes Dev.* **20,** 1511-1524.
Nguyen, L., Malgrange, B., Breuskin, I., Bettendorff, L., Moonen, G., Belachew, S. and Rigo, J. M. (2003). Autocrine/paracrine Activation of the GABA(A) Receptor Inhibits the Proliferation of Neurogenic Polysialylated Neural Cell Adhesion Molecule-Positive (PSA-NCAM+) Precursor Cells from Postnatal Striatum. *J. Neurosci.* **23,** 3278-3294.
Nieto, M., Schuurmans, C., Britz, O. and Guillemot, F. (2001). Neural bHLH Genes Control the Neuronal Versus Glial Fate Decision in Cortical Progenitors. *Neuron* **29,** 401-413.
Nordstrom, U., Jessell, T. M. and Edlund, T. (2002). Progressive Induction of Caudal Neural Character by Graded Wnt Signaling. *Nat. Neurosci.* **5,** 525-532.
Ohnuma, S. and Harris, W. A. (2003). Neurogenesis and the Cell Cycle. *Neuron* **40,** 199-208.
Ohnuma, S., Philpott, A. and Harris, W. A. (2001). Cell Cycle and Cell Fate in the Nervous System. *Curr. Opin. Neurobiol.* **11,** 66-73.
Ohtani, N., Goto, T., Waeber, C. and Bhide, P. G. (2003). Dopamine Modulates Cell Cycle in the Lateral Ganglionic Eminence. *J. Neurosci.* **23,** 2840-2850.
Okafuji, T., Funahashi, J. and Nakamura, H. (1999). Roles of Pax-2 in Initiation of the Chick Tectal Development. *Brain Res. Dev. Brain Res.* **116,** 41-49.
Olsen, S. K., Li, J. Y., Bromleigh, C., Eliseenkova, A. V., Ibrahimi, O. A., Lao, Z., Zhang, F., Linhardt, R. J., Joyner, A. L. and Mohammadi, M. (2006). Structural Basis by which Alternative Splicing Modulates the Organizer Activity of FGF8 in the Brain. *Genes Dev.* **20,** 185-198.
Pacary, E., Heng, J., Azzarelli, R., Riou, P., Castro, D., Lebel-Potter, M., Parras, C., Bell, D. M., Ridley, A. J., Parsons, M. et al. (2011). Proneural Transcription Factors Regulate Different Steps of Cortical Neuron Migration through Rnd-Mediated Inhibition of RhoA Signaling. *Neuron* **69,** 1069-1084.
Parras, C. M., Schuurmans, C., Scardigli, R., Kim, J., Anderson, D. J. and Guillemot, F. (2002). Divergent Functions of the Proneural Genes Mash1 and Ngn2 in the Specification of Neuronal Subtype Identity. *Genes Dev.* **16,** 324-338.
Patient, R. K. and McGhee, J. D. (2002). The GATA Family (Vertebrates and Invertebrates). *Curr. Opin. Genet. Dev.* **12,** 416-422.

Pattyn, A., Goridis, C. and Brunet, J. F. (2000). Specification of the Central Noradrenergic Phenotype by the Homeobox Gene Phox2b. *Mol. Cell. Neurosci.* **15,** 235-243.
Pattyn, A., Simplicio, N., van Doorninck, J. H., Goridis, C., Guillemot, F. and Brunet, J. F. (2004). Ascl1/Mash1 is Required for the Development of Central Serotonergic Neurons. *Nat. Neurosci.* **7,** 589-595.
Peltopuro, P., Kala, K. and Partanen, J. (2010). Distinct Requirements for Ascl1 in Subpopulations of Midbrain GABAergic Neurons. *Dev. Biol.* **343,** 63-70.
Peng, C. Y., Yajima, H., Burns, C. E., Zon, L. I., Sisodia, S. S., Pfaff, S. L. and Sharma, K. (2007). Notch and MAML Signaling Drives Scl-Dependent Interneuron Diversity in the Spinal Cord. *Neuron* **53,** 813-827.
Pevny, L. and Placzek, M. (2005). SOX Genes and Neural Progenitor Identity. *Curr. Opin. Neurobiol.* **15,** 7-13.
Pevny, L. H., Sockanathan, S., Placzek, M. and Lovell-Badge, R. (1998). A Role for SOX1 in Neural Determination. *Development* **125,** 1967-1978.
Pfaff, S. L. and Macfarlan, T. (2009). Transcriptional Networks and the Spinal Cord. In *Developmental Neurobiology* (ed. G. Lemke), pp. 172-177. Academic Press: Elsevier.
Pinard, A., Seddik, R. and Bettler, B. (2010). GABAB Receptors: Physiological Functions and Mechanisms of Diversity. *Adv. Pharmacol.* **58,** 231-255.
Pleasure, S. J., Anderson, S., Hevner, R., Bagri, A., Marin, O., Lowenstein, D. H. and Rubenstein, J. L. (2000). Cell Migration from the Ganglionic Eminences is Required for the Development of Hippocampal GABAergic Interneurons. *Neuron* **28,** 727-740.
Polleux, F., Whitford, K. L., Dijkhuizen, P. A., Vitalis, T. and Ghosh, A. (2002). Control of Cortical Interneuron Migration by Neurotrophins and PI3-Kinase Signaling. *Development* **129,** 3147-3160.
Powell, E. M., Campbell, D. B., Stanwood, G. D., Davis, C., Noebels, J. L. and Levitt, P. (2003). Genetic Disruption of Cortical Interneuron Development Causes Region- and GABA Cell Type-Specific Deficits, Epilepsy, and Behavioral Dysfunction. *J. Neurosci.* **23,** 622-631.
Powell, E. M., Mars, W. M. and Levitt, P. (2001). Hepatocyte Growth factor/scatter Factor is a Motogen for Interneurons Migrating from the Ventral to Dorsal Telencephalon. *Neuron* **30,** 79-89.
Pozas, E. and Ibanez, C. F. (2005). GDNF and GFRalpha1 Promote Differentiation and Tangential Migration of Cortical GABAergic Neurons. *Neuron* **45,** 701-713.
Prakash, N. and Wurst, W. (2004). Specification of Midbrain Territory. *Cell Tissue Res.* **318,** 5-14.
Puelles, E., Acampora, D., Gogoi, R., Tuorto, F., Papalia, A., Guillemot, F., Ang, S. L. and Simeone, A. (2006). Otx2 Controls Identity and Fate of Glutamatergic Progenitors of the Thalamus by Repressing GABAergic Differentiation. *J. Neurosci.* **26,** 5955-5964.
Puelles, E., Annino, A., Tuorto, F., Usiello, A., Acampora, D., Czerny, T., Brodski, C., Ang, S. L., Wurst, W. and Simeone, A. (2004). Otx2 Regulates the Extent, Identity and Fate of Neuronal Progenitor Domains in the Ventral Midbrain. *Development* **131,** 2037-2048.
Puelles, L. and Rubenstein, J. L. (2003). Forebrain Gene Expression Domains and the Evolving Prosomeric Model. *Trends Neurosci.* **26,** 469-476.
Rex, M., Orme, A., Uwanogho, D., Tointon, K., Wigmore, P. M., Sharpe, P. T. and Scotting, P. J. (1997). Dynamic Expression of Chicken Sox2 and Sox3 Genes in Ectoderm Induced to Form Neural Tissue. *Dev. Dyn.* **209,** 323-332.
Saarimaki-Vire, J., Peltopuro, P., Lahti, L., Naserke, T., Blak, A. A., Vogt Weisenhorn, D. M., Yu, K., Ornitz, D. M., Wurst, W. and Partanen, J. (2007). Fibroblast Growth Factor Receptors Cooperate to Regulate Neural Progenitor Properties in the Developing Midbrain and Hindbrain. *J. Neurosci.* **27,** 8581-8592.

Sandberg, M., Kallstrom, M. and Muhr, J. (2005). Sox21 Promotes the Progression of Vertebrate Neurogenesis. *Nat. Neurosci.* **8,** 995-1001.
Sato, T., Araki, I. and Nakamura, H. (2001). Inductive Signal and Tissue Responsiveness Defining the Tectum and the Cerebellum. *Development* **128,** 2461-2469.
Scholpp, S., Delogu, A., Gilthorpe, J., Peukert, D., Schindler, S. and Lumsden, A. (2009). Her6 Regulates the Neurogenetic Gradient and Neuronal Identity in the Thalamus. *Proc. Natl. Acad. Sci. U. S. A.* **106,** 19895-19900.
Scholpp, S. and Lumsden, A. (2010). Building a Bridal Chamber: Development of the Thalamus. *Trends Neurosci.* **33,** 373-380.
Scholpp, S., Wolf, O., Brand, M. and Lumsden, A. (2006). Hedgehog Signalling from the Zona Limitans Intrathalamica Orchestrates Patterning of the Zebrafish Diencephalon. *Development* **133,** 855-864.
Sessa, A., Mao, C. A., Hadjantonakis, A. K., Klein, W. H. and Broccoli, V. (2008). Tbr2 Directs Conversion of Radial Glia into Basal Precursors and Guides Neuronal Amplification by Indirect Neurogenesis in the Developing Neocortex. *Neuron* **60,** 56-69.
Shimojo, H., Ohtsuka, T. and Kageyama, R. (2008). Oscillations in Notch Signaling Regulate Maintenance of Neural Progenitors. *Neuron* **58,** 52-64.
Shimojo, H., Ohtsuka, T. and Kageyama, R. (2011). Dynamic Expression of Notch Signaling Genes in Neural stem/progenitor Cells. *Front. Neurosci.* **5,** 78.
Shivdasani, R. A., Mayer, E. L. and Orkin, S. H. (1995). Absence of Blood Formation in Mice Lacking the T-Cell Leukaemia Oncoprotein Tal-1/SCL. *Nature* **373,** 432-434.
Shivdasani, R. A. and Orkin, S. H. (1996). The Transcriptional Control of Hematopoiesis. *Blood* **87,** 4025-4039.
Simeone, A. (2000). Positioning the Isthmic Organizer Where Otx2 and Gbx2meet. *Trends Genet.* **16,** 237-240.
Smith, E., Hargrave, M., Yamada, T., Begley, C. G. and Little, M. H. (2002). Coexpression of SCL and GATA3 in the V2 Interneurons of the Developing Mouse Spinal Cord. *Dev. Dyn.* **224,** 231-237.
Soriano, P. (1999). Generalized lacZ Expression with the ROSA26 Cre Reporter Strain. *Nat. Genet.* **21,** 70-71.
Stancik, E. K., Navarro-Quiroga, I., Sellke, R. and Haydar, T. F. (2010). Heterogeneity in Ventricular Zone Neural Precursors Contributes to Neuronal Fate Diversity in the Postnatal Neocortex. *J. Neurosci.* **30,** 7028-7036.
Stoykova, A., Treichel, D., Hallonet, M. and Gruss, P. (2000). Pax6 Modulates the Dorsoventral Patterning of the Mammalian Telencephalon. *J. Neurosci.* **20,** 8042-8050.
Sugiyama, S., Funahashi, J. and Nakamura, H. (2000). Antagonizing Activity of Chick Grg4 Against Tectum-Organizing Activity. *Dev. Biol.* **221,** 168-180.
Sun, Y., Hu, J., Zhou, L., Pollard, S. M. and Smith, A. (2011). Interplay between FGF2 and BMP Controls the Self-Renewal, Dormancy and Differentiation of Rat Neural Stem Cells. *J. Cell. Sci.* **124,** 1867-1877.
Sussel, L., Marin, O., Kimura, S. and Rubenstein, J. L. (1999). Loss of Nkx2.1 Homeobox Gene Function Results in a Ventral to Dorsal Molecular Respecification within the Basal Telencephalon: Evidence for a Transformation of the Pallidum into the Striatum. *Development* **126,** 3359-3370.
Takahashi, T., Nowakowski, R. S. and Caviness, V. S.,Jr. (1995). The Cell Cycle of the Pseudostratified Ventricular Epithelium of the Embryonic Murine Cerebral Wall. *J. Neurosci.* **15,** 6046-6057.
Tamamaki, N., Yanagawa, Y., Tomioka, R., Miyazaki, J., Obata, K. and Kaneko, T. (2003). Green Fluorescent Protein Expression and Colocalization with Calretinin, Parvalbumin, and Somatostatin in the GAD67-GFP Knock-in Mouse. *J. Comp. Neurol.* **467,** 60-79.

Taverna, E. and Huttner, W. B. (2010). Neural Progenitor Nuclei IN Motion. *Neuron* **67,** 906-914.
Tiveron, M. C., Rossel, M., Moepps, B., Zhang, Y. L., Seidenfaden, R., Favor, J., Konig, N. and Cremer, H. (2006). Molecular Interaction between Projection Neuron Precursors and Invading Interneurons Via Stromal-Derived Factor 1 (CXCL12)/CXCR4 Signaling in the Cortical Subventricular zone/intermediate Zone. *J. Neurosci.* **26,** 13273-13278.
Tsunekawa, N., Yanagawa, Y. and Obata, K. (2005). Development of GABAergic Neurons from the Ventricular Zone in the Superior Colliculus of the Mouse. *Neurosci. Res.* **51,** 243-251.
Tyzio, R., Represa, A., Jorquera, I., Ben-Ari, Y., Gozlan, H. and Aniksztejn, L. (1999). The Establishment of GABAergic and Glutamatergic Synapses on CA1 Pyramidal Neurons is Sequential and Correlates with the Development of the Apical Dendrite. *J. Neurosci.* **19,** 10372-10382.
Uchikawa, M., Kamachi, Y. and Kondoh, H. (1999). Two Distinct Subgroups of Group B Sox Genes for Transcriptional Activators and Repressors: Their Expression during Embryonic Organogenesis of the Chicken. *Mech. Dev.* **84,** 103-120.
van Doorninck, J. H., van Der Wees, J., Karis, A., Goedknegt, E., Engel, J. D., Coesmans, M., Rutteman, M., Grosveld, F. and De Zeeuw, C. I. (1999). GATA-3 is Involved in the Development of Serotonergic Neurons in the Caudal Raphe Nuclei. *J. Neurosci.* **19,** RC12.
van Eekelen, J. A., Bradley, C. K., Gothert, J. R., Robb, L., Elefanty, A. G., Begley, C. G. and Harvey, A. R. (2003). Expression Pattern of the Stem Cell Leukaemia Gene in the CNS of the Embryonic and Adult Mouse. *Neuroscience* **122,** 421-436.
Vieira, C., Pombero, A., Garcia-Lopez, R., Gimeno, L., Echevarria, D. and Martinez, S. (2010). Molecular Mechanisms Controlling Brain Development: An Overview of Neuroepithelial Secondary Organizers. *Int. J. Dev. Biol.* **54,** 7-20.
Vue, T. Y., Aaker, J., Taniguchi, A., Kazemzadeh, C., Skidmore, J. M., Martin, D. M., Martin, J. F., Treier, M. and Nakagawa, Y. (2007). Characterization of Progenitor Domains in the Developing Mouse Thalamus. *J. Comp. Neurol.* **505,** 73-91.
Wadman, I. A., Osada, H., Grutz, G. G., Agulnick, A. D., Westphal, H., Forster, A. and Rabbitts, T. H. (1997). The LIM-Only Protein Lmo2 is a Bridging Molecule Assembling an Erythroid, DNA-Binding Complex which Includes the TAL1, E47, GATA-1 and Ldb1/NLI Proteins. *EMBO J.* **16,** 3145-3157.
Waite, M. R., Skaggs, K., Kaviany, P., Skidmore, J. M., Causeret, F., Martin, J. F. and Martin, D. M. (2012). Distinct Populations of GABAergic Neurons in Mouse Rhombomere 1 Express but do Not Require the Homeodomain Transcription Factor PITX2. *Mol. Cell. Neurosci.* **49,** 32-43.
Wang, D. D. and Kriegstein, A. R. (2009). Defining the Role of GABA in Cortical Development. *J. Physiol.* **587,** 1873-1879.
Wang, D. D., Krueger, D. D. and Bordey, A. (2003). GABA Depolarizes Neuronal Progenitors of the Postnatal Subventricular Zone Via GABAA Receptor Activation. *J. Physiol.* **550,** 785-800.
Wang, X., Tsai, J. W., LaMonica, B. and Kriegstein, A. R. (2011). A New Subtype of Progenitor Cell in the Mouse Embryonic Neocortex. *Nat. Neurosci.* **14,** 555-561.
Wassef, M. and Joyner, A. L. (1997). Early mesencephalon/metencephalon Patterning and Development of the Cerebellum. *Perspect. Dev. Neurobiol.* **5,** 3-16.
Wegner, M. and Stolt, C. C. (2005). From Stem Cells to Neurons and Glia: A Soxist's View of Neural Development. *Trends Neurosci.* **28,** 583-588.
Weiss, M. J., Yu, C. and Orkin, S. H. (1997). Erythroid-Cell-Specific Properties of Transcription Factor GATA-1 Revealed by Phenotypic Rescue of a Gene-Targeted Cell Line. *Mol. Cell. Biol.* **17,** 1642-1651.

Wilkinson, D. G. and Green, J. (1990). In Situ Hybridisation and the Threedimensional Reconstruction of Serial Sections. in Postimplantation Mammalian Embryos : A Practical Approach.
Willett, R. T. and Greene, L. A. (2011). Gata2 is Required for Migration and Differentiation of Retinorecipient Neurons in the Superior Colliculus. *J. Neurosci.* **31,** 4444-4455.
Wilson, S. I. and Edlund, T. (2001). Neural Induction: Toward a Unifying Mechanism. *Nat. Neurosci.* **4 Suppl,** 1161-1168.
Wozniak, R. J., Keles, S., Lugus, J. J., Young, K. H., Boyer, M. E., Tran, T. M., Choi, K. and Bresnick, E. H. (2008). Molecular Hallmarks of Endogenous Chromatin Complexes Containing Master Regulators of Hematopoiesis. *Mol. Cell. Biol.* **28,** 6681-6694.
Xia, Y., Brown, L., Yang, C. Y., Tsan, J. T., Siciliano, M. J., Espinosa, R.,3rd, Le Beau, M. M. and Baer, R. J. (1991). TAL2, a Helix-Loop-Helix Gene Activated by the (7;9)(q34;q32) Translocation in Human T-Cell Leukemia. *Proc. Natl. Acad. Sci. U. S. A.* **88,** 11416-11420.
Yamada, Y., Pannell, R., Forster, A. and Rabbitts, T. H. (2000). The Oncogenic LIM-Only Transcription Factor Lmo2 Regulates Angiogenesis but Not Vasculogenesis in Mice. *Proc. Natl. Acad. Sci. U. S. A.* **97,** 320-324.
Zhang, P. (1999). The Cell Cycle and Development: Redundant Roles of Cell Cycle Regulators. *Curr. Opin. Cell Biol.* **11,** 655-662.
Zhou, Y., Yamamoto, M. and Engel, J. D. (2000). GATA2 is Required for the Generation of V2 Interneurons. *Development* **127,** 3829-3838.
Zhu, Y., Li, H., Zhou, L., Wu, J. Y. and Rao, Y. (1999). Cellular and Molecular Guidance of GABAergic Neuronal Migration from an Extracortical Origin to the Neocortex. *Neuron* **23,** 473-485.