Regulation of GABAergic neuron identity and diversity in the developing midbrain

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Why walk, if you can run?

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by their roman numerals. In addition, unpublished data is included.
Due to a later change of name, the candidate (Kaia Achim) is referred to as Kaia Kala in the author lists of publications.

I Kaia Kala, Maarja Haugas, Kersti Lilleväli, Jordi Guimera, Wolfgang Wurst, Marjo Salminen and Juha Partanen. 2009. Gata2 is a tissue-specific post-mitotic selector gene for midbrain GABAergic neurons. Development 136: 253-62.

II Paula Peltopuro, Kaia Kala and Juha Partanen. 2010. Distinct requirements for *Ascl1* in subpopulations of midbrain GABAergic neurons. Developmental Biology 343: 63-70.

III Kaia Kala, James Li, Marjo Salminen and Juha Partanen. (2010) Distinct developmental origin for GABAergic neurons associated with dopaminergic nuclei in the ventral midbrain. Manuscript.

ABSTRACT

Gamma-aminobutyric acid (GABA) is the most abundant inhibitory neurotransmitter in the vertebrate brain. In the midbrain, GABAergic neurons contribute to the regulation of locomotion, nociception, defensive behaviours, fear and anxiety, as well as sensing reward and addiction. Despite the clinical relevance of this group of neurons, the mechanisms regulating their development are largely unknown. In addition, their migration and connectivity patterns are poorly characterized.

This study focuses on the molecular mechanisms specifying the GABAergic fate, and the developmental origins of midbrain GABAergic neurons. First, we have characterized the function of a zink-finger transcription factor Gata2. Using a tissue-specific mutagenesis in mouse midbrain and anterior hindbrain, we showed that Gata2 is a crucial determinant of the GABAergic fate in midbrain. In the absence of Gata2, no GABAergic neurons are produced from the otherwise competent midbrain neuroepithelium. Instead, the Gata2-mutant cells acquire a glutamatergic neuron phenotype. Ectopic expression of Gata2 was also sufficient to induce GABAergic differentiation in the chicken midbrain. Second, we have analyzed the midbrain phenotype of mice mutant for a proneural gene Ascl1, and described the variable and region-dependent requirements for Ascl1 in the midbrain GABAergic neurogenesis. These studies also have implications on the origin of distinct anatomical and functional GABAergic subpopulations in midbrain. Third, we have identified unique developmental properties of GABAergic neurons that are associated with the midbrain dopaminergic nuclei, the substantia nigra pars reticulata (SNpr) and ventral tegmental area (VTA). Namely, the genetic regulation of GABAergic fate in these cells is distinct from the rest of midbrain. In accordance to this phenomenon, our detailed fate-mapping analyses indicated that the SNpr-VTA GABAergic neurons are generated outside midbrain, in the neuroepithelium of anterior hindbrain.

ABBREVIATIONS

AP	anteroposterior
β-gal	beta-galactosidase
bHLH	basic domain helix-loop-helix
CNS	central nervous system
DA	dopaminergic
DCN	deep cerebellar nuclei
DV	dorsoventral
E	days of embryonic development
EGF	epidermal growth factor
FGF	fibroblast growth factor
FOG	Friend of GATA
GABA	gamma aminobutyric acid
GABAn	GABAergic neurons
HD	homeodomain
IC	inferior colliculus
IHC	immunohistochemistry
INM	interkinetic nuclear migration
ISH	in situ hybridization
IsO	isthmic organizer
LDTn	laterodorsal tegmental nucleus
LGE	lateral ganglionic eminence
MB	midbrain
MGE	medial ganglionic eminence
MHB	midbrain-hindbrain border
mRF	midbrain reticular formation
MZ	marginal zone
PAG	periaqueductal gray
PFC	prefrontal cortex
PPTn	pedunculopontine tegmental nucleus
R1	rhombomere 1
RMTg	rostromedial tegmental nucleus
SC	superior colliculus
Shh	Sonic hedgehog
SN	substantia nigra
SNpc	SN pars compacta
SNpr	SN pars reticulata
SVZ	subventricular zone
TF	transcription factor
Tx	tamoxifen
vl/d/dlPAG	ventrolateral/dorsal/dorsolateral PAG
vMB	ventral MB
VTA	ventral tegmental area
VZ	ventricular zone
Wnt	Wingless-related
wt	wild-type

1. Review of literature

1.1. Signal transduction in the central nervous system (CNS)

In the neuronal networks, signals are transmitted by passing on electrical currents within single cells. In contrast, cell-to-cell communication is primarily mediated by chemical signalling in specialized structures called synapses. The molecules used in synaptic signal transduction, neurotransmitters, are released by an activated presynaptic cell and can either potentiate (excitation) or prevent (inhibition) electrical activation in another, postsynaptic cell, which possesses receptors for these neurotransmitters. In the silent neurons, cell membrane is polarized at a resting membrane potential of ca -65 mV. This resting potential is established by maintaining different concentrations of electrically charged ions inside and outside of the cells. The most relevant in synaptic signalling are the differences in Na^+, K^+ and Cl^- concentrations so that the Na^+ and Cl^- concentrations are maintained higher outside while K^+ concentration is higher inside the cell.

During signalling at the synapse, neurotransmitters bind to their receptors, which either directly (in case of ionotropic receptors) of indirectly (metabotropic receptors) causes opening of transmembrane ion channels selective for one or several of the ions mentioned above, as well as Ca^{2+}. While the ionotropic receptors are ligand-gated ion channels, whose activity directly depends on the neurotransmitter binding, the metabotropic receptors are typically G-protein coupled transmebrane proteins. Neurotransmitter binding to the metabotropic receptor activates a downstream intracellular signalling cascade that can then result in opening of transmembrane ion channels and/or the release of Ca^{2+} from intracellular storages, thereby changing membrane potential. Opening of the transmembrane channels allows ions to flow across neuronal membrane, down their concentration gradients, bringing the membrane closer to electrical equilibrium potential for that particular ion. This results in membrane depolarization in the case of Na^+ or Ca^{2+} and hyperpolarization for K^+ or Cl^- flow.

1.2. GABA mediated signalling in the adult CNS

GABA is the main inhibitory neurotransmitter in adult mammalian CNS. Inhibition is crucial for the normal brain function, coordinating and balancing excitatory activity. Inhibitory effect of GABA on neuronal signalling is mediated by the GABA receptors that in essence prevent depolarization of the neuronal membrane, lowering basal activity of the neuron. There are two types of GABA receptors: ionotropic GABA-A and GABA-C receptors are voltage gated Cl^- channels that upon binding GABA allow Cl^- ions to flow inside the cell; while activation of metabotropic, G-protein coupled GABA-B receptors results in opening of K^+ channels and thus prevents depolarization by allowing K^+ efflux (Inoue et al., 1985; Turner and Whittle, 1983).

Inhibitory neurons producing GABA are found in almost all regions of the CNS. Despite the rather standard synaptic effects they mediate, these cells are highly variable in their gene expression, morphology, projection patterns and functional output (see overview by Petilla Interneuron Nomenclature Group et al., 2008). GABAergic circuits are at the basis of processes affecting sleep and wakefulness, epilepsy, anxiety, bipolar disorder and addiction, to name just few examples. Disruption of GABAergic

neurotransmission, for example by mutagenesis in experimental animals, typically results in spontaneous seizures, increased sensitivity to pain, and various behavioural abnormalities (Schuler et al., 2001).

1.3. GABA and developing neurons

The functions of GABA are quite different, and more varied during development, exceeding from the synaptic neurotransmission that it mediates in mature circuits. Importantly, the postsynaptic response to GABA signals in the immature brain, as opposed to the mature networks, can be also excitatory, meaning that the GABA-induced Cl^- currents exert a depolarizing effect on the developing neurons (Ben-Ari et al., 1989; Serafini et al., 1995). This opposite effect arises due to the differences in the transmembrane ionic balance in developing versus mature neurons. In immature neurons, the intracellular Cl^- concentration is higher than in the mature neurons. This is due to the presence of Na-K-2Cl cotransporter (NKCC1) in the neuronal membrane, which imports Cl^- ions (Khirug et al., 2008), but lack of a simultaneously acting Cl^- extrusion system. In such conditions, electrochemical gradient drives Cl^- ions out of the cell, making the Cl^- current depolarizing. During postnatal development, as neurons acquire more mature membrane properties, the ionic balance is changed so that the effect of GABA is switched from depolarizing to hyperpolarizing (Figure 1A, Rivera et al., 2005). This change in the balance of Cl^- ions correlates with the appearance of a Cl^--extruding cotransporter, potassium-chloride-cotransporter-2 (KCC2) that starts to be expressed in neuronal cell membrane during the first two postnatal weeks in rats (Rivera et al., 1999). The depolarizing (excitatory) activity of GABA during development is thought to be important for circuit formation, by generating synchronous activity in developing neuronal networks (giant depolarizing potentials; Ben-Ari et al., 1989; Sipila et al., 2005), but also modulating other aspects in circuit formation, like neuronal migration. The effect of GABA on cortical neuron migration has been shown to be concentration dependent and mediated by intracellular Ca^{2+} (Behar et al., 1996) and has also been demonstrated in vivo (Heck et al., 2007).

In addition to its facilitator role in developing neuronal networks, signalling via GABA-A receptors has been implicated in the regulation of proliferation in embryonic and neural stem cells, as well as regulation of cell numbers and growth of early embryos. This function seems to be directly linked to cell cycle progression, as GABA receptor activation leads to rapid activation of histone variant H2AX, a component of post-DNA replication checkpoint complex (Andang et al., 2008). GABA-induced depolarizing Cl^- currents have been shown to inhibit the DNA synthesis in neuronal progenitors also in later stages of development, using embryonic cortical slices. In contrast to stem cells, the effect on proliferation at later embryonic stages seems to be associated with the increase in intracellular Ca^{2+} (LoTurco et al., 1995).

In conclusion, GABA appears to serve as a multifunctional signalling agent in the developing brain, regulating proliferation, differentiation, gene expression (including GABA receptors themselves), migration, circuit formation and synaptogenesis (Figure 1B; reviewed in Ben-Ari, 2002; Ben-Ari et al., 2007; Owens and Kriegstein, 2002; Wang and Kriegstein, 2009).

1.4. GABAergic neurons in the midbrain

Alike in the CNS in general, GABAergic neurons are found widespread in the midbrain. However, specific spatial and functional subgroups can clearly be distinguished: in superior and inferior colliculi (collectively known as midbrain tectum), periaqueductal gray (PAG), midbrain reticular formation (mRF) and substantia nigra pars reticulata (SNpr). In the dorsal midbrain, GABA neurotransmission is involved in the visual information processing in superior colliculi (SC) (Binns and Salt, 1997). Well-studied examples of SC functions are the saccadic eye movements and multisensory and motor processing (King, 2004; McHaffie et al., 2005). The inferior colliculi (IC) are an important centre of auditory processing, and contain also a substantial number of GABAergic neurons which, besides local processing, contribute to tectothalamic outputs (Ito et al., 2009). In the PAG, GABAergic neurons constitute the main active circuit, providing constant tonic inhibition on all pathways in this region, controlling fear and anxiety, the related autonomic regulation and vocalizations, pain processing, nociception, and sexual behaviour (Behbehani, 1995). Distinct defensive behaviours can be functionally linked to distinct subdivisions of PAG: activation of more dorsal regions triggers active defence, so-called “fight-or-flight” behaviours and increase in heart rate, while ventral subdivisions mediate passive protective response, freezing and hypotension. In addition, dorsal PAG (dPAG), integrated with deep layers of SC and IC, is involved in processing aversive stimuli, fear associated with novel cues, and anxiety (Brandao et al., 1999). The GABAergic neurons in ventrolateral (vl) PAG are especially important in conditioned fear response (Brandao et al., 2005; Brandao et al., 2008); and together with midbrain reticular formation also regulate sleep and wakefulness (Fort et al., 2009; Sapin et al.,

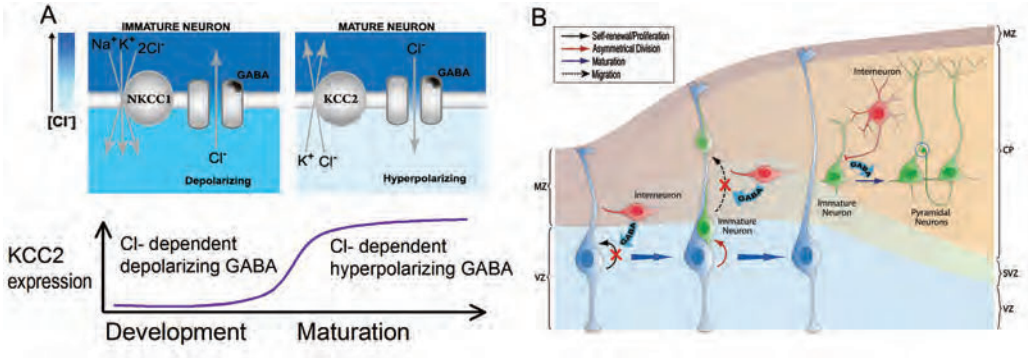

Figure 1. A, Opposite effects of Cl^- channel opening to membrane potential in developing and mature neurons. The switch from depolarizing to hyperpolarizing effect of Cl^- currents during development correlates with the upregulation of Cl-extruding pump KCC2. B, GABA signals are involved in multiple steps of neuronal differentiation. GABA-induced depolarizing currents suppress proliferation and promote neurogenesis. Depolarizing GABA signals promote cortical interneuron migration, while hyperpolarization provides a signal to stop migration and integrate in the cortex. GABA signalling also promotes neuronal maturation and establishment of connectivity. See text (chapter 1.3.) for more detailed examples. [Cl^-], chloride concentration; CP, cortical plate; MZ, marginal zone; VZ, ventricular zone; SVZ, subventricular zone. Adapted from Blaesse et al., 2009; Rivera et al., 2005; Wang and Kriegstein, 2009.

2009). The midbrain reticular formation contains a considerable number of loosely organized GABAergic neurons that are functionally poorly described.

1.4.1. VTA and SN GABAergic neurons

The most ventral GABAergic neurons found in midbrain are associated with midbrain dopaminergic nuclei in substantia nigra (SN) and VTA. The functions of dopaminergic (DA) neurons in VTA and SN pars compacta (SNpc) have been extensively studied, as they are an important part of the movement regulation pathways of basal ganglia (in SNpc) as well as regulators of mood and motivation (in VTA). In addition to the DA neurons, there is a substantial number of GABAergic cells in the ventral midbrain nuclei, which have not received the same enthusiastic attention. Nevertheless, the GABAergic neurons in these areas are a major group of targets for both neuropharmacology (Foster and Kemp, 2006) as well as drugs of abuse (Laviolette and van der Kooy, 2004b; Tan et al., 2010).

In the SN, GABAergic cells are located in its ventrolateral part, the pars reticulata, which is almost completely composed of GABAergic projection neurons. SNpr constitutes the major output centre of the basal ganglia, with efferent connections to thalamus, superior colliculus and brainstem, as well as local axon collaterals to dopaminergic neurons in SNpc and thus SNpr shares an important function in initiation, planning and execution of motor behaviour (Deniau et al., 2007; Tepper and Lee, 2007). In VTA, approximately one-third of the cells are GABAergic (Carr and Sesack, 2000). These cells contribute to the local circuits, regulating the activity of VTA DA neurons, but also constitute projection neurons to prefrontal cortex (PFC), nucleus accumbens and several limbic areas, the circuits underlying positive reward and conditioned learning processes (Fields et al., 2007). A highly interesting GABAergic neuron population has recently been identified in the caudal portion of VTA, termed “tail of VTA” or rostromedial tegmental nucleus (RMTg). The GABAergic neurons in RMTg are activated in response to psychostimulants, like cocaine, and preferentially project on VTA DA neurons (Kaufling et al., 2010). Further analysis has shown an important role of RMTg in encoding aversive stimuli, and the behavioural responses to such stimuli by modulating DA neuron activity in the VTA (Jhou et al., 2009a). Consistently to this function, RMTg receives afferents from other brain areas associated with aversive stimuli and reward processing, like lateral habenular nucleus. In addition, both afferent and efferent projections link RMTg with several brainstem regions associated with emotional processing, behaviour and learning (Jhou et al., 2009b).

Biochemically diverse phenotypes can be distinguished within both SNpr (Gonzalez-Hernandez and Rodriguez, 2000) and VTA GABAergic neurons (Olson and Nestler, 2007), and these subdivisions can have distinct functional properties (reviewed in Deniau et al., 2007; Fields et al., 2007).

1.5. Neuronal diversity and fate specification in CNS

There are large number of distinct neuronal subtypes in the CNS, classified by their morphology, projection patterns, electrical and chemical (neurotransmitter) properties. Neuronal cells of different phenotype can be generated from either spatially distinct progenitor populations or, alternatively, from the same region, but during different

time periods. Aside the enormous diversity that stems from a relatively homogenous progenitor population, the overall number of cells in CNS is also greatly expanded during the embryonic development. Both the expansion in cell numbers (proliferation of progenitors) and the differentiation of neurons (neurogenesis) take place simultaneously in the developing brain. Therefore, to eventually achieve a brain of correct size and morphological and functional composition, tight control over the proliferation and differentiation of developing neuronal precursors is essential.

1.5.1. Development and organization of the neural tube

All the neurons and glia in the adult brain are ultimately derived from the multipotent neuronal stem cells/progenitors forming the neuroepithelium, which constitutes the walls of the embryonic structure called the neural tube. The neural tube is a tubular structure formed by folding of the neuroepithelial tissue established at gastrulation (Beddington and Robertson, 1999). With the gastrulation, the cells of the embryo are segregated into the three germ layers (ecto-, meso- and endoderm), and the anteroposterior (AP) axis is established. At the end of gastrulation, part of the mesoderm cells aggregate to form a tubular condensate, the notochord, which serves to define the midline of the embryo. The neural tissue is specified from the midline ectoderm in response to the instructive signals provided from the embryonic primary organizer and other surrounding tissues

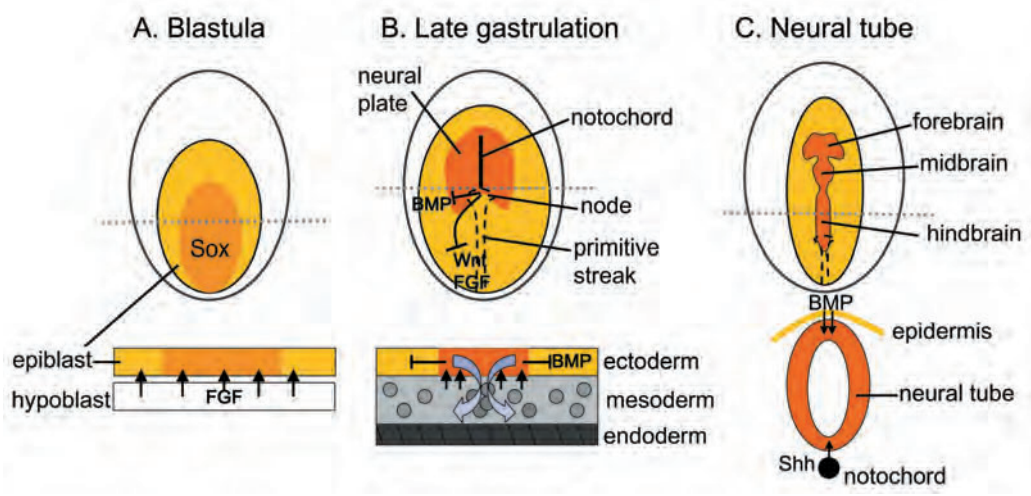

Figure 2. Neural induction and neural tube formation. Schematic representation of chicken embryos; upper images: dorsal view, bottom images: cross-section, the section plane is shown with the dotted line on upper images. A, Prior the gastrulation (blastula stage), prospective neural tissue can be identified by the Sox3 expression in the epiblast. Sox proteins are required for the competence to respond to further inductive signals, like Fibroblast growth factor (FGF) from the underlying hypoblast. B, During gastrulation, the node serves as the primary embryonic organizer for neural tissue, providing inhibitors of BMP (Bone morphogenetic protein) and Wnt signalling. Black arrows indicate the inductive signalling events. Grey block arrows in the lower panel indicate the ingression of prospective mesodermal cells during gastrulation. C, Neural plate folds to form the neural tube; fore-, mid- and hindbrain vesicles are indicated. Neural tube patterning is regulated by dorsalizing factors (BMP) emanating from the dorsal epidermis, and ventralizing factors (Sonic Hedgehog, Shh) from the notochord.

(reviewed by Saxen, 1989; Stern, 2005; Vieira et al., 2010) (Figure 2A,B). However, the competence to respond to these instructive signals may already be encoded in the presumptive neuroepithelial cells prior gastrulation (by the expression of Sox proteins, see 1.5.3a.) (Wilson and Edlund, 2001). The induced neuroepithelial sheet (neural plate) invaginates and folds to form the neural tube, which, as it grows, segregates into the major brain compartments: fore-, mid-, and hindbrain and spinal cord (Figure 2C). The following development of different compartments is controlled by signals from local secondary organizers typically established at compartmental interfaces (reviewed by Echevarria et al., 2003).

1.5.2. Patterning of the neural tube

Neuroepithelial cells are patterned already at the progenitor stage by combinatorial expression of a multitude of transcription factors (TFs) in regionally restricted patterns, which gives cells unique identities depending on their exact position in the neural tube. Vertebrate spatial patterning genes typically contain homeobox sequences that encode for the homeodomain motifs necessary for DNA recognition and the regulation of target gene expression.

1.5.2a. Anterior-posterior patterning of the neural tube

Neuroepithelial cells acquire distinct identities along the anteroposterior (AP) axis at the gastrulation stage, instructed by signals from adjacent non-neuronal tissues (Figure 2). Graded Wingless-related (Wnt) activity in combination with FGF signals appear to be sufficient to segregate the neural plate cells into the major AP compartments in consequent order (Nordstrom et al., 2002; Figure 3A). In addition, these early patterning events seem to encode the competence of cells to form the secondary signalling centre at the midbrain-hindbrain border (MHB) (Olander et al., 2006), the isthmic organizer (IsO).

In developing CNS, the earliest genes expressed differentially and in a regionally restricted manner along the AP axis are homeodomain (HD) TFs Otx2 and Gbx2, bordering at the midbrain (MB) and anterior hindbrain (rhombomere 1, r1), respectively. The expression of Otx2 and Gbx2 ultimately defines the MB and r1 territories and is crucial for the positioning of the IsO at their interface (Broccoli et al., 1999; Hidalgo-Sanchez et al., 2005; Millet et al., 1999). Subsequently, a set of intercellular signalling molecules are expressed within the neural tube. Fgf8 and Wnt1 released from IsO act as master regulators in patterning the MB-r1 territory (reviewed by Nakamura et al., 2005). IsO-derived signalling molecules selectively control the expression of, as well as receive regulatory feedback from a multitude of HD transcription factors. For instance, Fgf8 induces the expression of En1, En2 and Gbx2, and represses Otx2 and Pax6 expression to regulate the morphogenesis of midbrain and cerebellum structures (Puelles et al., 2004). The capacity of Fgf8 to induce these distinct structures, however, depends on previously established competence of the signal-receiving cells. For example, the instructing signals from Fgf8 seem to require Otx2 to be properly interpreted into positional information in the midbrain cells (Puelles et al., 2004). In the hindbrain, the expression of Irx2 in r1 precedes the formation of IsO. Later, modulation of Irx2 activity by IsO-derived Fgf8 is required for the development of cerebellum, the major derivative of dorsal r1 (Figure

3B; Matsumoto et al., 2004). Upon FGF-mediated activation, Irx2 is involved in the reorganization of Otx2, Gbx2 and Pax2 expression and thus in the fine positioning of IsO (Matsumoto et al., 2004).

Another feedback regulation loop acting in both mb and r1 involves Fgf8, En and Pax TFs (Figure 3B). En proteins are involved in the activation of Pax5 and inhibition of Pax6 expression. At the same time, conserved paired-box binding sites have been found in En2 upstream enhancer, and these Pax binding sited are required for Fgf8 to maintain En2 expression in MB-r1 region (Liu and Joyner, 2001). This regulation is important for maintaining En2 expression after its initial onset, which is independent of Pax (Liu and Joyner, 2001). Pax2-mediated regulation of Brn1 (Pou3f3) expression could provide feedback inhibition of Fgf8 in the MHB, restricting its expression strictly to anterior-most hindbrain and preventing it in more caudal cells (Simon et al., 2005).

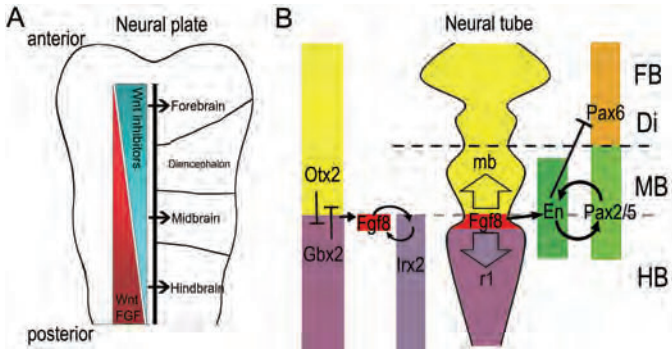

Figure 3. Interactions of major morphogens and transcription factors involved in the anteroposterior (AP) patterning of midbrain-rhombomere 1 area. A, Crude AP patterning of the neural plate by FGF and Wnt gradients at gastrulation. B, Positioning and patterning activities of the isthmic organizer (IsO). At first, Otx2 is expressed anterior and Gbx2 posterior to prospective mid-hindbrain boundary. Otx2 and Gbx2 cross-antagonize each other and establish the IsO area at their expression border, which will express Fgf8. The Fgf8 expression and IsO region is further refined by interactions of Fgf8 and pre-patterning transcription factor Irx2. Fgf8 signal from the IsO is required for the development of midbrain (MB)-rhombomere 1 (R1) structures. Fgf8 signalling from IsO induces the expression of En and Pax 2/5, which form a positive feedback loop to promote each other's expression. In addition, En represses the forebrain (FB) determinant Pax6 expression posterior to diencephalon (Di)-midbrain border.

1.5.2b. Dorsal-ventral patterning of the neural tube

Concerning the cellular diversity within a single brain compartment, the DV patterning is of notable importance. The central morphogens in basal midbrain and r1 patterning are Sonic hedgehog (Shh) that is released from the floor plate (Agarwala et al., 2001; Ericson et al., 1995); bone morphogenetic proteins (BMP) from the overlying ectoderm and Wnt signals from the roof plate (Wurst and Bally-Cuif, 2001). In the midbrain, Shh establishes distinct DV domains by activating its downstream transcriptional activator Gli2 in the ventrolateral midbrain and restricting the Gli3 repressor function to the lateral-dorsal areas (Figure 5A) (Blaess et al., 2006).

The actions of Shh in neuroepithelial patterning and the specification of neuronal subtypes are best exemplified in the development of spinal cord. During the patterning of the ventral spinal cord, Shh is released from the notochord and floor plate (Marti et al, 1995). Graded Shh signalling then regulates the expression of an array of target

genes that pattern and specify distinct populations of ventral neurons. Shh target genes, which typically encode HD family transcriptional repressors, can respond to certain levels of signalling, and they can either be repressed (class I) or activated (class II) by Shh (Briscoe et al., 2000; Ericson et al., 1997). Shh target genes are initially expressed in broad domains, which are thereafter rapidly refined. This refinement involves cross-repression between class I and class II Shh targets at the domain boundaries (Briscoe et al., 2000). Thus, in the ventral spinal cord, graded Shh morphogen activity is translated into a HD TF code that defines distinct progenitor populations separated by sharp domain boundaries (Figure 4; reviewed in Briscoe and Novitch, 2008; Briscoe, 2009). Subsequently, the expression of proneural genes follows these boundaries, and distinct neuronal fates arise from each progenitor domain. In the neuronal subtype specification, HD proteins can act synergistically with the proneural gene function. For example, bridging the LIM-HD TFs Lhx3 and Isl1 to the proneural protein NeuroM via an adaptor protein NLI increases the efficiency of ventral motor neuron differentiation (Lee and Pfaff, 2003). This interaction provides a direct link between a HD code and neuronal fate specification in the neural tube.

Similar model of HD code of neuronal progenitors has recently been depicted for mouse midbrain (Nakatani et al., 2007). In this model, at least seven distinct DV domains can be identified based on their expression of distinct combinations of HD proteins Lmx1a, Foxa2 (HNF3β), Nkx2-2, Nkx6-1 and Pax7 (Figure 5A). The function of Shh in the establishment of midbrain HD protein expression has been extensively studied in chicken model, where Shh can induce the full ventral gene expression pattern, so-called “midbrain arcs”, in correct orientation respective to the Shh source (Agarwala et al., 2001), and also repress dorsal gene expression (Bayly et al., 2007).

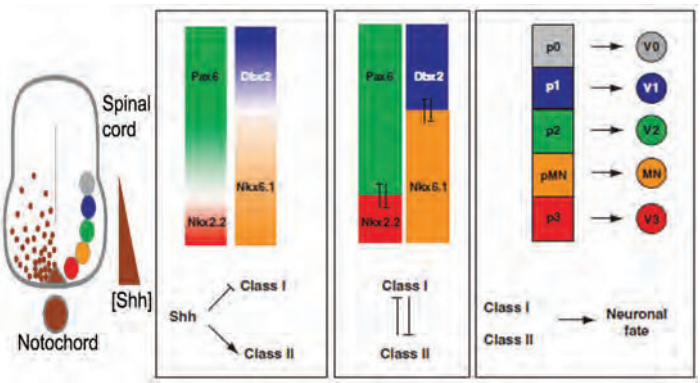

Figure 4. DV patterning in the ventral spinal cord is controlled by Shh gradient. Shh is released from the notochord and floor plate. Shh represses the expression of its Class I target genes, while inducing the Class II targets. Subsequently, Class I and II transcription factors counter-repress each other, refining distinct progenitor domain boundaries in ventral spinal cord. Each progenitor domain expresses characteristic set of Shh target genes and gives rise to distinct neuronal subtypes. p0-3, interneuron progenitor domains along the DV axis. V0-3, distinct classes of interneurons derived from the respective progenitor domains. pMN, motor neuron progenitor domain; MN, ventral spinal cord motor neurons. Adapted from Briscoe, 2009.

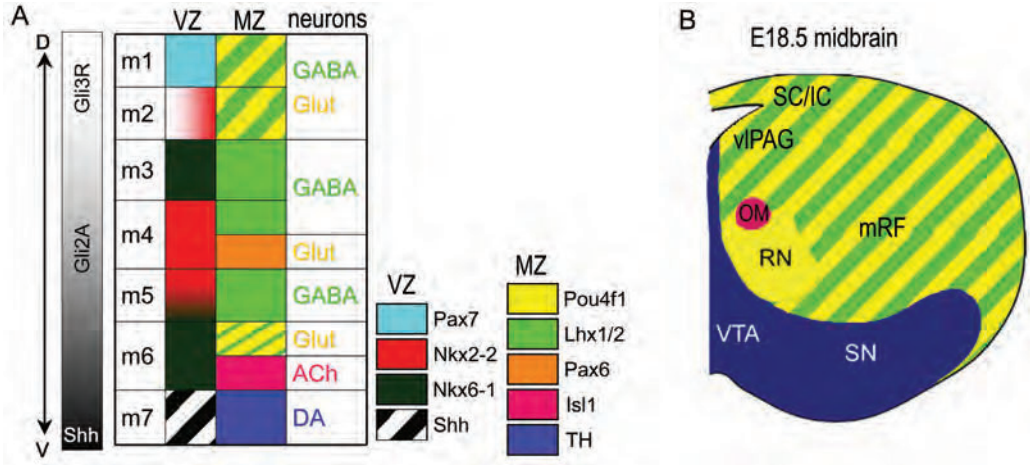

Figure 5. Dorsoventral homeodomain (HD) protein code in the vertebrate midbrain can be associated with distinct neuronal subtypes generated in each domain. A, HD transcription factor code is established by graded Shh signalling, which is translated into changing levels of Gli2 activator (A) and Gli3 repressor (R) along the DV axis. Seven DV domains (m1-m7) can be distinguished in the midbrain neuroepithelium. Characteristic expression of patterning genes in m1-m7 is indicated for the ventricular zone (VZ; progenitor cells) and marginal zone (MZ; postmitotic neurons). Neuronal subtypes arising from each midbrain domain are also indicated. B, Schematic representation of prenatal (E18.5) mouse midbrain showing the neuronal nuclei originating from midbrain neuroepithelium. Modified from Puelles, 2007.

Later in development, neurons born from the distinct DV domains can be characterized by the expression of Nkx2-2, Nkx6-1, Pou4f1, Pax6 or Lhx1/2 (Nakatani et al., 2007). The HD protein expression can also be linked with different midbrain nuclei later arising from these DV domains (Agarwala and Ragsdale, 2002; Bayly et al., 2007) and neuronal subtypes within these nuclei (Figure 5B; reviewed by Puelles, 2007).

1.5.2c. Integration of AP and DV patterning in midbrain

IsO-derived molecules alone as well as in various combinations are involved in highly integrated AP and DV patterning in the midbrain and r1 neuroepithelium. For example, the cellular competence in responding Shh signals can be modulated by Otx2 (Puelles et al., 2004), Fgf8 and Wnt1 (Joksimovic et al., 2009b; Prakash and Wurst, 2006). Otx2 has also been implicated in the restriction of Shh expression domain, thus affecting the development of ventral midbrain dopaminergic and glutamatergic neurons (Puelles et al., 2003). Later in development, Otx2 may also regulate neuronal subtype selection via activation of proneural proteins (Vernay et al., 2005). Thus, a complex and recursively wired patterning network is established in the neural tube early in development, specifying the competence of the individual progenitor cells in different position (reviewed in Prakash and Wurst, 2004; Wurst and Bally-Cuif, 2001).

1.5.3. Regulation of neurogenesis

Neuroepithelial cells within the neural tube are well organized according to their cell cycle progression and differentiation status. The proliferative neuroepithelial cells are

lined near the lumen (apical side) where they form a layer called the ventricular zone (VZ). Differentiating cells exit the cell cycle, i.e. become postmitotic, lose connection with the luminal surface and move into the outer layer called the marginal zone (MZ), along their basal process. The proliferative cells in ventricular zone possess connections with both the apical and basal surfaces of the neural tube. The position of the nucleus of these highly elongated cells depends on the stage of cell cycle: The cell division (i.e. mitosis and cytokinesis) takes place at the apical surface. During the G1 phase the nuclei migrate towards the basal side of the VZ, where the DNA replication (S phase) takes place. Finally, during G2 the nuclei move back apically. This movement, called interkinetic nuclear migration (INM), might be coupled with neurogenesis as, apparently, certain cell fate selection factors (e.g. Notch receptors and targets, see 1.5.3c) seem to be expressed in higher levels at the apical than in basal side of ventricular zone (Henrique et al., 1997; Lindsell et al., 1996). Consistently, disturbances in INM can affect the proliferation of neuronal progenitors and prolonged stay in the basal region, where Notch signalling is less active, can trigger premature differentiation (Del Bene et al., 2008). Furthermore, the Notch mRNA has been shown to be stabilized in G2/M/G1 phase and less stable in S-phase cells (Cisneros et al., 2008). Oscillations in the mRNA levels of Notch target genes have also earlier been observed in dividing neuroepithelial cells (Hirata et al., 2002).

In addition to Notch, FGF and Epidermal growth factor (EGF) signalling is also important for the neuronal stem cell identity. In the neuroepithelium, FGF signalling promotes undifferentiated status in neuronal progenitors (Akai et al., 2005; Bertrand et al., 2000). In the absence of FGF signalling, the neuronal progenitors in ventral midbrain undergo premature cell cycle exit, resulting in the depletion of progenitor pool and the loss of midbrain ventral cell types, like DA neurons (Saarimaki-Vire et al., 2007).

1.5.3a. Neuronal progenitor identity: SoxB1 family

The earliest known markers of neural identity in a developing embryo are the Sox (SRY-like HMG box) TFs of the SoxB1 subgroup, Sox1-3. Already prior gastrulation, Sox2 is expressed in the presumptive neuroectoderm, where it contributes to the responsiveness to the neural induction by FGF (Mizuseki et al., 1998) and inhibitors of BMP (Kishi et al., 2000). Shortly after gastrulation, the expression of Sox2 and Sox3 becomes restricted to the forming neural tissue (Rex et al., 1997; Wood and Episkopou, 1999). In the neural tube, Sox1-3 expression maintains the undifferentiated status of neural progenitors (Bylund et al., 2003; Graham et al., 2003), and this function appears independent or complementary to that of Notch signalling (Holmberg et al., 2008). While the Notch target Hes prevents neurogenesis by repressing proneural gene expression, Sox3 is able to prevent differentiation even in the presence of a proneural protein Ngn2 (Bylund et al., 2003). While Sox1-3 preserve progenitor status, neurogenesis is promoted the transcriptional repressors of SoxB2 subgroup, Sox14 and Sox21 (Uchikawa et al., 1999). The SoxB1 and SoxB2 TFs apparently regulate a similar set of target genes, and the co-expression of Sox21 and Sox3 can have different effect to the neurogenesis in the chicken neural tube, depending on the ratio of the factors (Sandberg et al., 2005). Thus, for the neurogenesis to occur, the SoxB1 activity must be counteracted by SoxB2 subgroup TFs.

***1.5.3b. Commitment to neuronal fate**: proneural genes*

Neuronal differentiation is thought to be triggered by so-called proneural genes. Vertebrate proneural genes code for basic domain-helix-loop-helix (bHLH) transcription factors that can instruct neuroepithelial cells to exit cell cycle and commit to a neuronal fate (Lee et al., 1995). To elicit their function, bHLH proteins form a heterodimeric complex with ubiquitously expressed E-proteins, typically E2A gene variants E12 and E47 in neurons. The proneural bHLH-E-protein complex binds DNA at a consensus sequence, CANNTG, generally known as E-box, and thus activates the expression of genes related to neuronal differentiation (Massari and Murre, 2000). In addition to directing cells to postmitotic neuronal fate in general, at least in some cases proneural genes seem to be coupled with certain neuronal fates. For example, Ascl1 drives the GABAergic, and Ngn2 the glutamatergic neuron differentiation during the mammalian cortex development (Fode et al., 2000; Parras et al., 2002). The ability of proneural TFs to drive neuronal subtype commitment may, at least in part, be regulated in the level of the target gene activation, either via the binding partners of proneural proteins or by physical interaction with the DNA. Although the bHLH-E-protein complex is capable of DNA binding, it may not be sufficient to initiate transcription. Typically, cofactors are needed for the target gene activation, and variation in the expression and/or activity of these factors can give cells different competence to respond to certain proneural signal (Lee and Pfaff, 2003). Enhancers can exhibit different binding affinities to different transcription factors, even if these are very closely related (Castro et al., 2006). Thus, proneural proteins can exert their function in diverse manners, depending on their cofactors, transcriptional regulatory regions of the target genes and the pre-patterned competence of the cell (reviewed in Guillemot, 2007; Powell and Jarman, 2008).

1.5.3c. Maintenance of the progenitor pool: lateral inhibition

As proneural factors trigger differentiation, it is crucial to simultaneously maintain a certain pool of stem cells until the complete spectra of neurons have been generated. To avoid depletion of the progenitor pool, the differentiating neurons are utilizing a lateral inhibition mechanism mediated by Notch signalling. Notch signalling cascade is triggered by the proneural proteins, which induce the expression of Notch ligand, Delta, on the cell surface (Kunisch et al., 1994). Delta can then interact with Notch receptor on the neighbouring cells, triggering proteolytic cleavage of Notch and nuclear translocation of the Notch intracellular domain (Bray, 2006). Nuclear Notch intracellular domain activates the transcription of target genes, including the bHLH transcriptional repressors of Hes family. Hes proteins efficiently repress proneural genes and maintain a proliferative “stem cell” state (Chen et al., 1997; Ishibashi et al., 1995; Kageyama et al., 2007). Therefore, the levels of Hes and thus also the levels of proneural activity in neuronal progenitors vary according to the levels of Delta expressed by the neighbouring cells. Considering also the link between Notch activity and INM (chapter 1.5.3), cells with different neurogenic potential can be spatially distinguished in VZ (Latasa et al., 2009): For one, the S-phase cells with basally localized nuclei express high levels of proneural genes and thus gain neurogenic competence. In the G1/M/G2 phase (cells with apically localized nuclei), this competence can be realized by neurogenic division and cell cycle exit. However, at the same time, the neurogenic potential can also be

efficiently suppressed, as the neuronal progenitors in the apical VZ receive higher levels of Notch signalling. This cycle makes the expression of proneural genes oscillatory and not an explicit indicator of neurogenesis for a particular cell (Kageyama et al., 2009). In short, proneural genes, via Delta-Notch signalling, induce a self-regulatory feedback loop that enables, through iterations and stochastic selection, to trigger neurogenic cell cycle exit in limited number of progenitors despite of their rather ubiquitous expression (Bertrand et al., 2002; Kageyama et al., 2005; Kageyama et al., 2007).

The functions of Notch signalling can exceed from its best-known role in lateral inhibition (reviewed by Cau and Blader, 2009). For instance, Notch has been implicated in binary fate decision in vertebrate CNS, during the excitatory versus inhibitory interneuron fate selection in mouse spinal cord (see chapter 1.6.3.).

1.5.3d. Neuronal subtype specification in proliferating progenitors

Although the expression of proneural genes can be spatially restricted, proneural activity generally activates pan-neuronal genes and further regulation of neuronal fate involves additional subtype specification factors. The subtype specification factors often, but not exclusively belong to bHLH family, and these TFs can both activate and repress particular target genes (Guillemot, 2007). For example in the ventral forebrain, Ascl1 and homeobox TF Dlx1/2 are independently expressed in the VZ and subventricular zone (SVZ), and required for different aspects of neurogenesis (Yun et al., 2002). While Ascl1 seems to regulate the timing and extent of the production of late progenitors or early precursors in the SVZ (Casarosa et al., 1999; Yun et al., 2002), the Dlx1/2 are required for the generation of postmitotic GABAergic interneurons from the SVZ progenitors (Anderson et al., 1997b; Yun et al., 2002).

In the midbrain, the expression of bHLH family transcriptional repressor Helt is induced independently of Ascl1 in the GABAergic progenitor domains (Miyoshi et al., 2004), and its expression is required for GABAergic neurogenesis (Guimera et al., 2006b). Apparently, Helt supports the GABAergic fate commitment by suppressing the glutamatergic neurogenesis (Nakatani et al., 2007) (discussed in more detail below).
Thus, in the neural tube, different classes of TFs must act synergistically to specify distinct progenitor domains and neuronal subtypes therein. In this cascade, both proneural genes and other bHLH TFs apparently lie downstream of the HD TF code (Guillemot, 2007).

1.5.3e. Subtype specification in postmitotic neurons

The subtype specification factors can act as sole promoters or inhibitors of certain cell fate, or function in both directions, driving multipotent neuronal precursors towards a specific subtype, simultaneously preventing expression of genes characteristic to other cell types. Such bimodal regulators are called selector genes; and a few of them have been characterized in the selection of GABAergic versus glutamatergic neuron phenotype in the mammalian CNS. For example, bHLH TF Ptf1a controls GABAergic as opposed to glutamatergic fate selection in both mammalian cerebellum and dorsal spinal cord (Glasgow et al., 2005; Hoshino et al., 2005; Pascual et al., 2007) (see 1.6.2a and 1.6.3a).

At the same time, HD proteins Tlx1 and Tlx3 are both required as well as sufficient for the induction of glutamatergic neurogenesis in the expense of GABAergic neurons in the dorsal spinal cord, thus acting as selectors for the glutamatergic fate (Cheng et al., 2004; Cheng et al., 2005).

In summary, combined expression of proneural, subtype specification and/or selector genes in the neuroepithelium enables different types of neurons to be produced in a simultaneous manner and contributes to the diversity of cell types found in the adult brain (Figure 6).

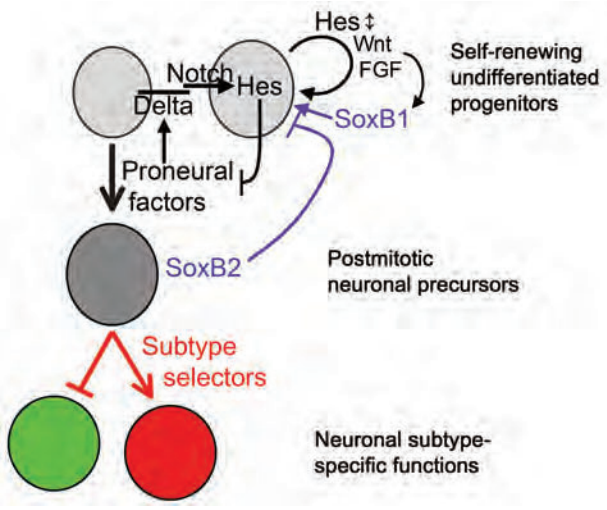

Figure 6. The genetic regulatory events controlling neurogenesis.
Neural progenitor identity and self-renewing status is maintained by FGF, Wnt and SoxB1 (blue) family TFs. Neurogenic cell cycle exit is triggered by proneural factors. Proneural factors also initiate lateral inhibition cascade via Delta-Notch signalling, promoting the maintenance of undifferentiated status in neighbouring cells. SoxB2 (blue) TFs suppress progenitor identity in the postmitotic neuronal precursors. Additional subtype specification factors (red) further refine the subtype identity of newborn neuronal precursors.

1.6. GABAergic neurogenesis

Preceding the neurogenesis, brain compartments are subjected to distinct regional patterning cues from the adjacent tissues as well as local signalling gradients (reviewed above). Therefore, it is perhaps not surprising that the cellular competence and neurogenic potential differs between the compartments. Indeed, the molecular mechanisms regulating the development of GABAergic neurons vary greatly between different brain regions (Table 1).

Table 1. Genes involved in the GABAergic neuron development in different brain regions.

Function / Brain region	Spatial patterning: HD proteins	Proneural: bHLH proteins	Progenitor specification: HD or bHLH	Postmitotic subtype specification: bHLH, GATA (midbrain)
Forebrain	Shh: Nkx2-1 (MGE) (Sussel et al., 1999) Gsh2 (LGE) (Corbin et al., 2000)	Ascl1 (Casarosa et al., 1999; Horton et al., 1999)	Dlx1/2 (Anderson et al., 1997a; Anderson et al., 1997b)	??
Midbrain	Shh: Nkx2-2, Nkx6-1, Pax7 (Agarwala et al., 2001; Blaess et al., 2006)	Ascl1 (Miyoshi et al., 2004) (II)	Helt (Guimera et al., 2006b; Nakatani et al., 2007)	Gata2 (I)
Cerebellum	IsO: En2 (Sgaier et al., 2007; Sillitoe et al., 2008)	(Ascl1, Ngn1/2) implications from fate map (Kim et al., 2008) and expression data (Zordan et al., 2008)	(Pax2) implications from expression data (Maricich and Herrup, 1999; Weisheit et al., 2006)	Ptf1a (Hoshino et al., 2005)
Dorsal spinal cord	Gsh1/2 (Mizuguchi et al., 2006)	Ascl1 (late neurogenesis) (Mizuguchi et al., 2006)	Pax2 (Cheng et al., 2004)	Ptf1a (Glasgow et al., 2005) Lbx1 (Cheng et al., 2005)
Ventral spinal cord	Shh: Nkx6-1, Irx3 (Briscoe et al., 2000)	Ascl1 (Li et al., 2005)	Gata2 (Zhou et al., 2000)	Tal1 (Muroyama et al., 2005)
mutant phenotype (mouse)	molecular and /or morphological mispatterning of the brain domain	delayed/diminished GABAergic neurogenesis	GABAergic neurons missing	GABAergic neurons missing + re-specification to glutamatergic fate

1.6.1. Forebrain

The GABAergic neuron development as a whole, starting from early specification in progenitor domains to proper migration and connectivity in the adult structures, is perhaps best understood in the mammalian forebrain. In the forebrain, GABAergic neurons contribute to two major compartments: first, they are an integral component of basal ganglia, which is central in motor coordination. Second, the inhibitory interneurons in the adult cortex and hippocampus are primarily GABAergic.

During forebrain development, the glutamatergic excitatory projection neurons are produced in the dorsal forebrain (also called pallium) and move radially to populate the

neocortical layers, while the inhibitory interneurons synthesizing GABA originate from the ventral forebrain neuroepithelium (subpallium) and migrate to the cortex tangentially (Marin and Rubenstein, 2001). The key molecules in regional patterning of ventral forebrain are HD proteins Nkx2-1, Nkx6-2, and Gsh2 and Dlx1/2, which establish genetically distinct subpallial regions (Figure 7A). Dlx1/2 also appear to be the main regulators of forebrain interneuron fate, as the Dlx1/2 mutant mice lack the GABAergic neurons from all derivatives of this brain region: striatum, cortex and olfactory bulbs (Anderson et al., 1997a; Anderson et al., 1997b). It has been shown in forebrain slice cultures that Dlx2 can activate the expression of Gad1 and Gad2, which are required for the function of GABAergic neurons (Stuhmer et al., 2002), as well as Arx, which contributes to their migration in the forebrain (Colasante et al., 2008).

The diversity of GABAergic neurons has also been first noticed in the forebrain. Apparently, genetically distinct subpallial progenitor pools give rise to specific subtypes of inhibitory neurons as characterized by their co-neurotransmitters (parvalbumin, somatostatin, calretinin, calbindin and neuropeptide Y) used alongside GABA (Flames and Marin, 2005; Fogarty et al., 2007). The cortical interneuron subtypes are specified at the time they become postmitotic, prior their migration (Fogarty et al., 2007), and different Dlx gene expression may contribute to their segregation, as different subtypes are affected in different extent in Dlx1 mutant mice (Cobos et al., 2005).

As already mentioned, the proneural TF Ascl1 is thought to be coupled with the cortical GABAergic neuron fate. Ascl1 is expressed throughout the medial and lateral ganglionic eminences (MGE/LGE) in subpallium (Horton et al., 1999). When ectopically

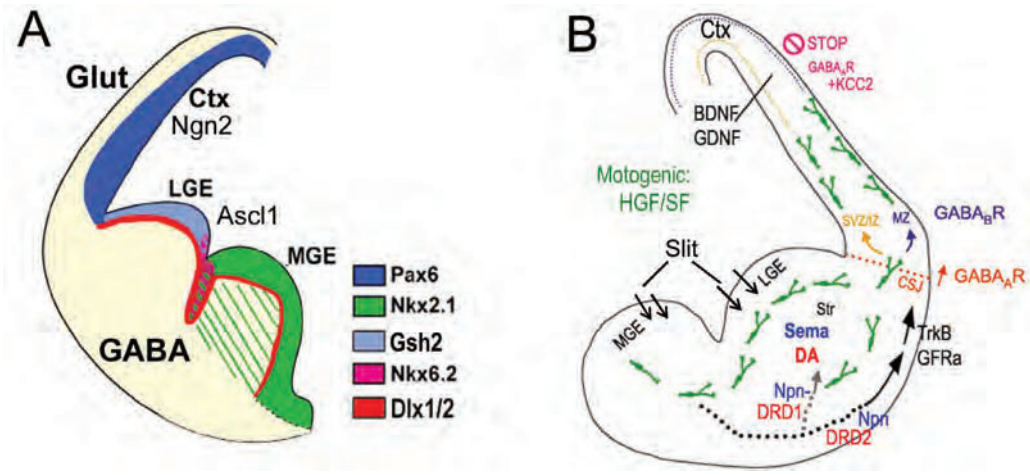

Figure 7. Dorsoventral patterning, neuronal subtypes and tangential migration of GABAergic interneurons in the forebrain. Schematic representations of E14.5 mouse forebrain (coronal view of one hemisphere). A, Expression of HD transcription factors and proneural genes. GABAergic (GABA) neurons are born in ventral forebrain (MGE/LGE), glutamatergic (Glut) neurons derive from dorsal neuroepithelium. B, Migration routes of GABAergic neurons during forebrain development. Selection of proteins known to regulate interneuron migration are indicated in approximate relevant locations. See chapter 1.6.1a. for further details. LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; Ctx, cortex, Str, striatum, DA, dopamine. Modified from Hernandez-Miranda et al., 2010; Heng et al., 2007.

expressed in pallial neuroepithelium, Ascl1 can induce the expression of GABAergic markers (Fode et al., 2000). However, in the Ascl1 deficient mice, GABAergic neurons can still be produced from the LGE, but not from MGE in the subpallial neuroepithelium (Casarosa et al., 1999). Therefore, Ascl1 seems to be sufficient, but only region-specifically required for GABAergic neurogenesis in forebrain. Indeed, Ascl1 might be required for the specification of certain subtypes of GABAergic neurons, while its more general function is to regulate the timing of the neurogenesis in the GABAergic progenitors via induction of Notch signalling (Casarosa et al., 1999; Yun et al., 2002). As proneural proteins drive neurogenic cell cycle exit, activate neuronal subtype specific target genes, and yet non-cell autonomously regulate the proliferation of adjacent progenitors, their function is undoubtedly complex. For example during the development of sympathetic chain neurons in the peripheral nervous system, Ascl1 is required in the early stage of neurogenesis, but this requirement can be overcome in time. It was postulated that other transcription factors can compensate for Ascl1, but only after reaching certain expression levels. At least in part, the roles of Ascl1 diverge from its proneural function in this context, since Ngn2 was not able to substitute for Ascl1 (Pattyn et al., 2006).

1.6.1a. Tangential migration of GABAergic neurons in the forebrain

The tangential migration in the cortex is well studied and shown to be regulated by wide variety of factors (Figure 7B; reviewed by Marin and Rubenstein, 2001; Marin and Rubenstein, 2003). Motogenic signals generally promote the interneuron migration. Such signals can be provided by growth factors, like Hepatocyte growth factor/Scatter factor (HGF/SF) that is expressed in the developing subpallium and cortical plate, adjacent to the interneuron migratory routes (Powell and Jarman, 2008). In the early phases of interneuron development, *Slit1* is expressed in the VZ and SVZ of the ganglionic eminences (Yuan et al., 1999), and Slit-mediated repulsion is thought to guide neurons away from the VZ where they were born, and towards more marginal regions, affecting all the subpallial neurons similarly (Andrews et al., 2007; Zhu et al., 1999).

Several examples of neurotrophin signalling guiding the subpallial interneurons towards cortex have been described. Neurotrophins BDNF and NT-4 seem to be released from the developing projection neurons in the pallium, and respective TrkB receptors are found in migrating interneurons. The activation of downstream signalling via TrkB receptors promotes both the motility and migration speed of cortical interneurons (Polleux et al., 2002). Another neurotrophin, GDNF, via GFR1α receptor signalling is similarly implicated in the tangential migration of MGE-derived GABAergic neurons (Pozas and Ibanez, 2005). In addition, analyses of TrkB and GFR1α mutant mice have revealed a possibility that neurotrophin signalling might selectively regulate the migration of certain cortical interneuron subtypes (Canty et al., 2009; Polleux et al., 2002). Upon reaching cortex, interneurons are distributed in different layers of the cerebral wall. The chemokine SDF-1 (stromal cell-derived factor-1) is expressed by the cortical progenitors (Tiveron et al., 2006) and implicated in the intercortical dispersion of GABAergic neurons, as the lack of SDF-1 or its receptor CXCR4 (expressed by the migrating subpallial neurons) results in abnormal distribution of GABAergic cells in the cortical layers (Lopez-Bendito et al., 2008; Tiveron et al., 2006). Apparently, also GABA signalling itself can regulate the migration of cortical interneurons. In different

concentrations, GABA can promote either directional or random migratory behaviour in cultured cells (Behar et al., 1996). Low levels of GABA are present in the developing forebrain (Cuzon et al., 2006). In cortical slices, depolarizing GABA-A signals promote interneurons to enter cortical plate (Cuzon et al., 2006), and enhance the migration within the cortical plate (Bortone and Polleux, 2009). The tangentially migrating MGE-derived GABAergic neurons express GABA-B receptor, and the blockade of GABA-B signalling in these cells results in alterations in their migratory route and accumulation in the deep cortical layers (Lopez-Bendito et al., 2003). Recently, it has been shown that hyperpolarizing signals via GABA-A receptors can trigger the termination of interneuron migration in the cortical plate. The capacity to respond such signals depends on the maturation of interneurons and consequent upregulation of KCC2, which is responsible for the switch from de- to hyperpolarizing response of GABA-A activation (Bortone and Polleux, 2009).

However, not all the interneurons born in ventral forebrain end up in the cortex, but proportion of them remain to populate the GABAergic nuclei of basal ganglia, including striatum. Repulsive guidance molecules Sema3A and Sema3F are expressed in striatum during cortical development, and thus only the interneurons lacking Sema receptors, Neuropilins, can enter the striatum, while the neurons targeted to cortex express Neuropilins and avoid the source of Sema (Marin et al., 2001). In addition, dopamine signalling has been shown to affect subpallial interneuron migration both positively, via dopamine receptor D1 activation, and negatively via D2 receptor activation (Crandall et al., 2007).

1.6.2. Rhombomere 1

The main derivatives of the anterior hindbrain or r1 are the dorsally derived cerebellum along with the deep cerebellar nuclei (DCN), a set of brainstem nuclei including the precerebellar complex, which provides mainly excitatory input to the cerebellum; mostly cholinergic laterodorsal (LDTn) and pedunculuopontine tegmental (PPTn) nuclei, and the serotonergic nuclei of dorsal raphe (Zervas et al., 2005). In addition, GABAergic neurons are found in many locations in the brainstem, but these populations are poorly characterized at present.

1.6.2a. Cerebellum

Cerebellum is a highly conserved region in the vertebrate brain, which main functions lay in the motor control and proprioception. There are two major neuronal subtypes in the cerebellum: the glutamatergic granule cells and GABAergic Purkinje, Golgi, basket and stellate cells. The DCN have both glutamate- and GABAergic component (Hoshino et al., 2005). Because of the relative simplicity of this system, the molecular regulation of cerebellar neurogenesis is relatively well characterized.

During development, cerebellum is derived from dorsal r1. The cerebellar GABAergic cells are born from the ventricular zone of dorsal r1 (Hoshino et al., 2005), while the glutamatergic cells of cerebellar cortex, DCN and precerebellar nuclei originate from the dorsocaudal edge of the fourth ventricle, a structure called the rhombic lip (Machold and Fishell, 2005; Rodriguez and Dymecki, 2000; Wang et al., 2005).

Cell lineage tracing has demonstrated that the expression of bHLH TF Ptf1a is restricted to the GABAergic lineage in the cerebellum. In accordance with this expression pattern, Ptf1a function is central for the GABAergic neurogenesis in the dorsal r1, as Ptf1a mutant mice lack all cerebellar GABAergic neurons, including those in the DCN (Hoshino et al., 2005). It has also been shown that ectopic Ptf1a can induce the differentiation of cerebellar GABAergic neuron-like cells in dorsal forebrain, confirming the master regulator properties of this gene (Hoshino et al., 2005). In the absence of Ptf1a, the presumptive GABAergic precursors apparently adopt a glutamatergic granule cell fate (Pascual et al., 2007). However, because of the loss of trophic support normally provided by the GABAergic cells, the granule cells undergo apoptosis, eventually resulting in a complete loss cerebellum in the Ptf1a mutant mice (Hoshino et al., 2005; Pascual et al., 2007).

1.6.2b. GABAergic neurons in the ventral r1

Although LDTn and PPTn are mostly cholinergic nuclei, they have been found to have significant GABAergic component (Boucetta and Jones, 2009; Wang and Morales, 2009). LDTn and PPTn have been implicated in locomotion, nociception, pain (Lee et al., 2000), but also, in connection to VTA, in addiction (Ishibashi et al., 2009) or reward (Laviolette and van der Kooy, 2004a). However, the function and origin of the presumed GABAergic component in LDTn and PPTn is unknown. The Math1- expressing cells in the rhombic lip contribute to the precerebellar system and also to the mesopontine LDTn and PPTn (Machold and Fishell, 2005), but the cell type of those rhombic lip derivatives has not been identified.

1.6.3. Spinal cord

Along the length of the spinal cord, two functional subdivisions are found at all levels: sensory relay circuit in dorsal part and the motor circuit in the ventral spinal cord. The inhibitory GABAergic neurons are integral part of both circuits, modulating both the sensory inputs to the CNS and the descending motor output, respectively.

1.6.3a. Dorsal spinal cord

In the dorsal spinal cord, GABAergic and glutamatergic progenitor domains are segregated by Notch signalling induced by the coordinate action of Ascl1 and HD family TFs Gsh1/2 (Mizuguchi et al., 2006). Subsequently, Ptf1a is specifically expressed in the postmitotic cells in the GABAergic precursor domain, where it instructs differentiation by repressing the dorsal glutamatergic determinant *Tlx3* (Glasgow et al., 2005; Mizuguchi et al., 2006). In the absence of Ptf1a, spinal cord dorsal horn sensory interneurons fail to activate the GABAergic differentiation markers Pax2, Lhx1/5 and *Gad1*, and instead are trans-specified into a glutamatergic phenotype characteristic to the cells born in their adjacent progenitor domains in neural tube (Tlx3^{+}, *Vglut2*$^{+}$). This fate switch demonstrates dual properties of Ptf1a: supporting the GABAergic and suppressing glutamatergic differentiation routes (Glasgow et al., 2005).

1.6.3b. Ventral spinal cord

Yet other transcriptional regulators are involved in GABAergic fate specification in the ventral spinal cord, where the progenitors of GABAergic (V2b; characterized by the

expression of Gata2, Gata3 and Tal1) and glutamatergic (V2a; Chx10^{+}) interneurons are closely related in lineage (Karunaratne et al., 2002; Smith et al., 2002; Zhou et al., 2000). This and adjacent progenitor domains are initially patterned by the Shh gradient and its target gene expression (Briscoe et al., 2000; Poh et al., 2002). Both GABA- and glutamatergic lineages arise from the Foxn4 and Ascl1 expressing p2 progenitor domain, which gives rise to V2 postmitotic precursors, characterized by the expression of Gata2 and Lhx3 (Karunaratne et al., 2002; Zhou et al., 2000). Early V2 precursors are subsequently segregated into two distinct groups by Foxn4-induced Notch1-Delta4 signalling, which initiates the expression of V2b fate determinant Tal1 (Del Barrio et al., 2007; Peng et al., 2007). The V2a/b segregation is further refined by the Lim-HD transcriptional cofactor Lmo4, which mediates the formation of Gata2-Tal1 transcriptional complex that in turn regulates Gata2/3 expression in V2b cells (Joshi et al., 2009). Gata2 as well as Tal1 have been shown to be sufficient for the commitment to V2b phenotype (Karunaratne et al., 2002; Muroyama et al., 2005; Zhou et al., 2000). At the same time, the loss of Tal1 results in specific lack of V2b neurons (Muroyama et al., 2005), while in the Gata2 mutant, the whole V2 precursor population fails to develop (Zhou et al., 2000).

1.6.4. GABAergic neurogenesis in the midbrain

The mechanisms of GABAergic development in the midbrain have been, surprisingly, ignored until quite recently. Nakatani et al (2007) studied the spatial patterning relevant to the GABAergic neurogenesis and identified seven distinct progenitor domains along the dorsoventral axis in midbrain neuroepithelium called m1-m7 (Figure 5) (Nakatani et al., 2007). From these domains, m3-m5 and, later in development also m1 and m2 give rise to GABAergic neurons.

Currently, the proneural gene Ascl1 (Miyoshi et al., 2004; II), bHLH-Orange domain transcriptional repressor Helt (Heslike, Megane; Guimera et al., 2006b; Nakatani et al., 2007) and zink-finger TF Gata2 (I) appear to be the only TFs implicated in the GABAergic fate specification in the mammalian midbrain. Both Ascl1 and Helt are expressed in the midbrain VZ regions associated with GABAergic neurogenesis (Miyoshi et al., 2004; Nakatani et al., 2007). Requirement of Ascl1 in the early development of GABAergic neurons has been proposed based on the absence of Gad1 (Gad67, Glutamic acid decarboxylase 1, essential enzyme for GABA biosynthesis) and GABA in the Ascl1 mutants at E11.5 (Miyoshi et al., 2004). However, this study was not focusing on Ascl1 function in detail and no later stages were analyzed for GABAergic neuron development. This study addressed also Helt function, and Helt overexpression was shown to promote GABAergic neurogenesis in Ascl1 expressing progenitors. Thus, Helt seems to act in concert with Ascl1 to specify GABAergic phenotype or regulate timing of GABAergic neurogenesis (Miyoshi et al., 2004). Analysis of Helt mutant mice has further elucidated the role of Helt in midbrain GABAergic neurogenesis, demonstrating requirement for it in certain subgroups of midbrain GABAergic neurons. Namely, the expression of GABAergic neuron markers *Gad1/2* is absent in superior and inferior colliculi, dPAG and some more lateral midbrain nuclei in these mice. The lack of gross anatomical defects indicates that in the absence of Helt, prospective GABAergic progenitors might re-specify to another neuronal subtype or undergo incomplete differentiation (Guimera

et al., 2006b). Indeed, further analyses have shown that in the Helt mutant midbrain, most of the regions associated with GABAergic neurogenesis seem to produce glutamatergic cells expressing Pou4f1 and Vglut2 (Nakatani et al., 2007). Nakatani et al (2007) also proposed the mechanism of Helt function, using gain- and loss-of-function studies. Because of the ectopic Ngn1/2 expression in the Helt-deficient VZ and, conversely, the downregulation of Ngn1/2 in the areas of Helt overexpression, they proposed that Helt directs the selection of GABAergic over glutamatergic fate via repression of Ngn1/2, which in turn promotes glutamatergic neurogenesis (Nakatani et al., 2007).

1.6.5a. Migration and maturation of midbrain GABAergic neurons

Current understanding of GABAergic neuron migration patterns in the midbrain is limited. Few studies concerning the migration of GABAergic neurons in the superior colliculi have indicated contribution of both radial and tangential migration (Tan et al., 2002; Tsunekawa et al., 2005). Thus far, predominantly radial distribution of tectal neurons has been observed after clonal labelling of cells in dorsal midbrain neuroepithelium. The contribution of tangential movements seems to be minor and might only contribute to the uniform distribution of different cell types in the later stages of development (Tan et al., 2002). Using *Gad67* promoter driven GFP, these migratory patterns were also directly observed in dorsal midbrain GABAergic neurons (Tsunekawa et al., 2005). This is in contrast to the tangential route undertaken by interneurons in the forebrain.

1.6.5b. Connectivity of midbrain GABAergic projection neurons

Midbrain GABAergic neurons project to several distant brain regions, and these projection patters have been described to some extent. By combining immunolabelling with retrograde and anterograde tracer analyses, GABAergic cells in the VTA have been found to send projections to PFC (Carr and Sesack, 2000) and nucleus accumbens (Van Bockstaele and Pickel, 1995). Recently, GABAergic tectothalamic projections originating from inferior colliculi have been described using similar approach (Ito et al., 2009).

The mechanisms of axon guidance and the establishment of connectivity patterns of midbrain GABAergic neurons is poorly understood, most likely due to the technical limitations, and lack of specific markers which would allow separation between different GABAergic subpopulations.

However, the expression of several axon guidance molecules has been described in ventral midbain (vMB), and their functions have been evaluated in respect to vMB DA projections. The expression of axon guidance molecule Netrin and its receptor DCC have been identified in the developing rat ventral midbrain and striatum (Livesey and Hunt, 1997) and the neurite growth in cultured vMB DA neurons has been shown to be regulated by Netrin1 and Slit2 (Lin et al., 2005). EphA5 and EphB1, receptors for repulsive guidance factors of Ephrin family, are also expressed in ventral midbrain. The expression of EphB1 in the SN neurons appears complementary to the EphrinB2 expression in the SN projection target areas in striatum. Consistently, Ephrin B2 could suppress neurite outgrowth in SN DA neurons in a co-culture system (Yue et al., 1999). The prefrontal projections from medial VTA express Neuropilin2 and are guided by Sema3 signals (Kolk et al., 2009). It is possible that at least some of these mechanisms are also involved in the development of GABAergic projections in ventral midbrain.

1.7. Serotonergic neurons in the r1

In the hindbrain, Shh, via its targets Nkx2-2 and Nkx6-1, establishes the HD code which defines the zone of, and is sufficient for, the serotonergic neurogenesis in the ventral neural tube (Craven et al., 2004; Pattyn et al., 2003). The Nkx2-2^{+} VZ progenitors give rise to Lmx1b^{+} serotonergic precursors. Lmx1b function is required for the expression of serotonergic markers Pet1 and 5-HT, but not Gata3 in the r1 (Ding et al., 2003). Proneural gene Ascl1 is expressed in the ventral r1, and appears to regulate both the neurogenesis and fate specification of the serotonergic neurons, controlling the expression of Lmx1b, Gata3, Pet1 and 5-HT (Pattyn et al., 2004). In addition, serotonergic neurons in r1 fail to develop in the Gata2 mutant neural tube explant cultures, indicating the requirement of Gata2 in these cells (Craven et al., 2004). Furthermore, the ability of Shh to induce serotonergic neuron differentiation depends on functional Gata2. Gata2 is also sufficient to induce ectopic serotonergic neuron development in r1, characterized by the expression of Lmx1b, Pet1 and 5-HT. Interestingly, this ability is restricted to r1 and does not extend to more caudal hindbrain, although the requirement for Gata2 still persists (Craven et al., 2004).

1.8. GATA transcription factors

GATA transcription factors are C4 zink-finger DNA-binding proteins that were first identified as direct regulators of globin gene transcription and have been since known as critical components of genetic cascades regulating hematopoiesis (Cantor and Orkin, 2002). Functionally, GATA proteins act as transcriptional regulators through a consensus sequence (A/T)GATA(T/G) (Lowry and Atchley, 2000). Altogether, there are 6 GATA transcription factors found in mammals, Gata1-6. Gata4-6 regulate the heart, smooth muscle and endoderm development. Gata1-3 are mostly known as hematopoietic regulators. Of these three factors, Gata2 is the most abundantly expressed in respective lineages and serves as an early and essential regulator of both hematopoietic and endothelial development (Patient and McGhee, 2002).

1.8.1. Gata2 and transcriptional regulation of hematopoietic lineages

As GATA family TFs are highly homologous, they are all able to recognize and bind efficiently the same consensus sequence. Nevertheless, GATA TFs can exert different and even opposing effects on the same targets and their functions are highly context-dependent (Wozniak et al., 2008). These differences may be explained to some extent by the use of different cofactors. Indeed, GATA factors typically function as a part of a multi-protein transcription regulatory complex. GATA proteins can interact with various partners: Friend of GATA (FOG) family proteins Fog1 and Fog2, bHLH TF Tal1(SCL)-E2A heterodimers, LIM-only TF Lmo2 and LIM-family cofactor NLI (Ldb1), Krüppel-like factors (Klf1), Ets family TF PU.1, and histone modifying proteins p300/CBP (reviwed by Cantor and Orkin, 2002; Ferreira et al., 2005). Transcriptional cofactors can modulate the function of GATA TFs. For example, in cultured blood cell progenitors, the presence or absence of Tal1 in the Gata2 complex can define the nature (i.e. activation or repression) of target gene regulation (Tripic et al., 2009). Similarly, Fog1, a cofactor directly interacting with Gata1, facilitates its DNA binding and thus mediates

the exchange of Gata2 for Gata1 in the promoters of their common, but differentially regulated targets (Letting et al., 2004; Pal et al., 2004).

As GATA TFs themselves, their transcriptional co-effectors have been discovered and extensively studied in the hematopoiesis. These studies revealed the principle of combinatorial regulation of hematopoietic target genes and the importance of coordinate functions of GATA TFs Gata1 or Gata2 together with bHLH TF Tal1. Probably the best-known GATA-Tal1 effector complex has been described by Wadman et al (1997) and consists of DNA-bound Gata1 or Gata2 and Tal1-E47, which are bridged by Lmo2 and NLI proteins (Figure 8A; Wadman et al., 1997). This complex recognizes a conserved

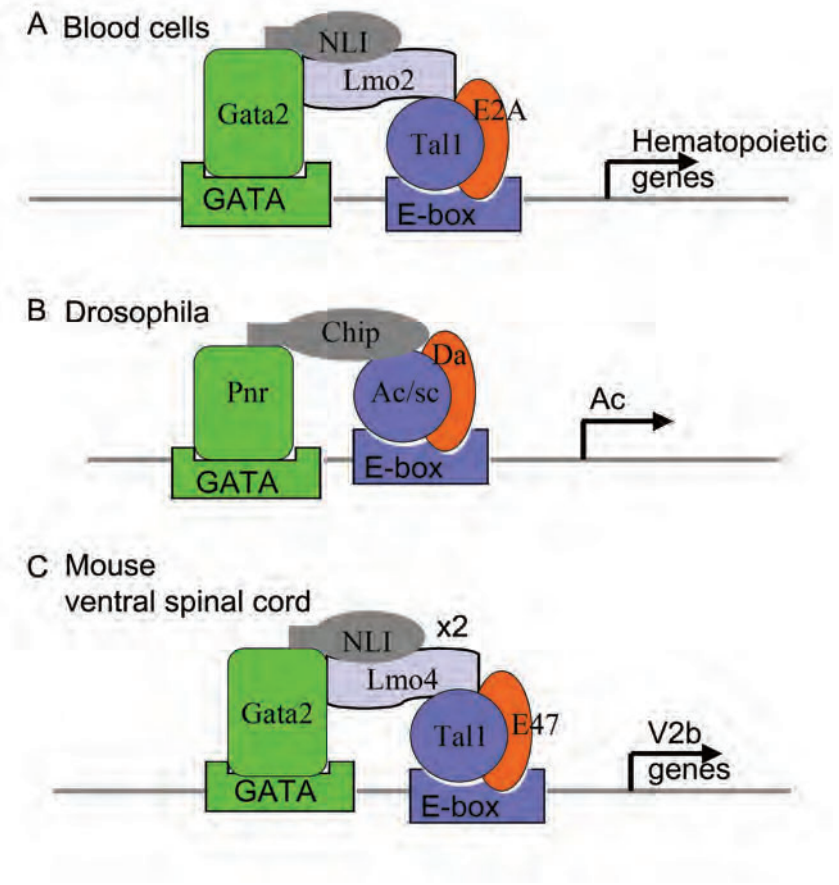

Figure 8. Similar GATA-E-box binding transcriptional regulatory complex is functional during human and mouse hematopoiesis, CNS development and Drosophila CNS. A, Gata2-Tal1/E2A-Lmo2-NLI complex is regulating hematopoietic gene expression in mouse and human erythroid cells. B, Heteromeric complex of Pannier (Pnr), Achaete-scute/Daughterless (Ac/sc, Da) and Chip involved in the autoregulation of Achaete (Ac) during Drosophila sensory bristle development. C, The Gata2-Tal1/E47-Lmo4-NLI complex is involved in the cell fate selection (V2b fate) in mouse ventral spinal cord interneurons. The bipartite complex consists of two such Gata2-Tal1/E47-Lmo4-NLI units, linked via NLI-NLI interaction. Schematics are based on Wadman et al., 1997; Ramain et al., 2000; Joshi et al., 2009.

GATA-E-box motif (Wozniak et al., 2008), which is found in the regulatory modules of many genes expressed in hematopoietic as well as endothelial cells (Cheng et al., 2009). Furhermore, the Gata-Tal1-E47-Lmo2-NLI complex has been shown to directly regulate the expression of several hematopoietic genes, like glycoprotein A (Lahlil et al., 2004), Lmo2 (Landry et al., 2009) and Runx2 (Nottingham et al., 2007). In addition, the E-box-GATA elements can be found from enhancers driving gene expression in other tissues, like Tal1 midbrain enhancer (Ogilvy et al., 2007).

1.8.2. Gata2 in the CNS

GATA TFs also have roles outside the hematopoietic system. Of the six GATA TF-s, Gata2 and Gata3 have been shown to be expressed in the vertebrate CNS (George et al., 1994; Groves et al., 1995; Nardelli et al., 1999). Elucidating the function of GATA TFs in the CNS has been hampered by the early lethality by E9.5-11.5 of the knockout animals due to severe defects in either blood (Gata2-null, Tsai et al., 1994) or heart development (Gata3-null, Pandolfi et al., 1995). Therefore, these mutations only allow analysis of relatively early steps in neurogenesis. Nevertheless, disorganization and reduced thickness of neuroepithelium was observed in the Gata3 mutant mice (Pandolfi et al., 1995), suggesting that GATA TFs can have distinct roles later in development, unrelated to their function in hematopoiesis.

Nardelli et al (1999) demonstrated the expression of Gata2 and Gata3 genes in the pretectum, ventral midbrain, hindbrain and spinal cord of the developing CNS and showed that Gata2 is required for Gata3 expression in these regions (Nardelli et al., 1999). The midbrain expression of Gata2 could be detected the earliest at E9.5, and Gata3 at E10.5 (Nardelli et al., 1999). However, the possible cell type specificity of Gata2 expression remained ambiguous in this study. More careful analysis of cell types expressing Gata2 has revealed its expression in the ventral spinal cord interneurons (Karunaratne et al., 2002; Zhou et al., 2000), and their GABAergic sublineage (Lundfald et al., 2007), serotonergic neurons in rostral hindbrain (Craven et al., 2004) and motor neuron precursors in the more caudal hindbrain, in rhomomere 4 (Gavalas et al., 2003; Pata et al., 1999). In all these locations, the general rule of the expression dynamics of GATA TFs is that Gata2 expression shortly precedes that of Gata3. The proteins can cross-regulate each other, supported by the presence of GATA binding sites in their promoters, a general feature for GATA family members (Burch, 2005). During neurogenesis, Gata2 seems to be expressed in the late progenitor/early differentiation stage, hinting to a function in cell type specification or neurogenic cell cycle exit.

The function of Gata2 in the context of cellular fate specification has indeed been demonstrated in various regions of the CNS. Gain- and loss-of-function studies have shown that Gata2 is both required and sufficient for ventral interneuron development in the spinal cord (Karunaratne et al., 2002; Zhou et al., 2000). Gata2 is also implicated in the development of r1 serotonergic neurons, as discussed above (chapter 1.7.). Functional analyses have revealed double GATA binding elements in the 5' proximal region of serotonergic fate determinant Pet1 and shown that Gata2 binding to these sites is required for Pet1 expression (Krueger and Deneris, 2008). Thus, the function of Gata2 in cell fate specification involves direct regulation of cell type specific gene expression.

It has been proposed that besides activation of cell type specific targets, Gata2 may also instruct withdrawal from cell cycle, at least in some biological context (El Wakil et al., 2006). In this study, forced expression of Gata2 could influence cell proliferation in the chicken spinal cord, as judged by reduction in CyclinD1 and up-regulation of cyclin-dependent kinase inhibitor p27 expression in Gata2-expressing cells. Also, downregulation of Notch1, Delta1 and Hes5 were observed upon Gata2 overexpression. This function was interpreted as independent of proneural activity, as the repression of Hes5 was observed also when proneural activity was repressed. (El Wakil et al., 2006)

1.8.3. Gata2 transcriptional complex in the CNS

The central Gata-Tal complex in blood progenitors is formed by DNA-bound Gata1 and Tal1-E-protein heterodimer(s), which are bridged by Lmo2 and NLI (see 1.8.1.). Interestingly, quite similar transcriptional complex is involved in the proneural patterning during the sensory bristle development in Drosophila (Ramain et al., 2000) (Figure 8B). Drosophila Gata homolog Pannier, proneural TFs Achaete-Scute (Ac/sc) and E2A homolog Daughterless (Da) are engaged into a transcriptional complex via protein-protein interactions with the bridging molecule Chip (similar to vertebrate Ldb/NLI). The Pannier-Ac/Sc-Da-Chip complex mediates the direct autoregulation of Ac/sc during sensory bristle development. Interestingly, U-shaped (Ush), homolog of vertebrate FOG proteins, is acting as inhibitor of this complex formation by binding Pannier (Ramain et al., 2000). In the mouse spinal cord, similar Gata2-Tal1-Lmo4-NLI complex (V2b complex) formation is required for the activation target genes and defining the GABAergic identity of V2b ventral interneurons (Joshi et al., 2009) (Figure 8C). In this study, also the enhancer elements containing bipartite GATA-E-box motifs were identified in both Gata2 and Gata3 genes and shown to be the target of V2b complex binding (Joshi et al., 2009).

1.8.3a. Protein partners of Gata2

Notably, the expression of both Gata2 and Tal1 has been described in the chick midbrain (Herberth et al., 2005) around the stages of GABAergic neuron production. Also, the expression of Tal1 (Elefanty et al., 1999) and Tal2 (Mori et al., 1999) have been shown in the mouse midbrain, raising the possibility that a GATA-Tal regulatory complex could also participate in neuronal fate selection in this brain region. Furthermore, the conditional deletion of Tal1 in the CNS with Nestin-cre has been shown to result in slight reduction of midbrain and more pronounced loss of hindbrain GABAergic neurons (Bradley et al., 2006). Also Tal2 has been implicated in the brain development, although no cell-type specific role was studied (Bucher et al., 2000).

Another group of Gata interactors in midbrain may be FOG proteins, which directly bind GATA TFs and can regulate their activity either positively or negatively (Cantor and Orkin, 2005). From this family, at least Fog2 is also expressed in the midbrain (Lu et al., 1999).

2. AIMS OF THE STUDY

As discussed above, Gata2 has been shown to regulate neuronal subtype specification in the developing CNS. Although the expression of Gata2 in the midbrain has been recognized, its cell type specificity and function in this brain region had not been addressed.

With this study, we aimed to:

I) characterize the cell lineages and developmental stages where Gata2 and Gata3 are expressed in the mouse midbrain and anterior hindbrain (r1);

II) investigate the function of Gata2 in the development of midbrain and r1;

III) study the function of Ascl1 in midbrain and r1 GABAergic neurogenesis;

IV) study the molecular pathways involved in the GABAergic neuron development in midbrain and

V) fate map the GABAergic neuron subpopulations in the mouse midbrain, focusing on the SNpr and VTA GABAergic neurons.

3. METHODS

Table 2. Methods used in this study

Method	Reference	Publication
BrdU labelling	see publications	I, II, III
PCR genotyping	Table 4	I, II, III
Whole-mount in situ hybridization (ISH)	(Henrique et al., 1995)	I, III
ISH on paraffin sections	(Wilkinson and Green, 1990)	I, II, III
Immunohistochemistry (IHC)	(Kala et al., 2008)	I, II, III
combined ISH and IHC	see below	I, III
Statictical methods	student's t-test (two-tailed)	I, III
In ovo electroporation	(Nakamura and Funahashi, 2001), see below	I
Tamoxifen administration	(Hayashi and McMahon, 2002)	III
X-gal staining	(Kala et al., 2008)	III

Combined ISH and IHC

ISH pre- and post- treatments were performed first, according to the modified protocol (Wilkinson and Green, 1990). After post-hybridization washes, samples were blocked in TNB blocking solution (TSA Biotin kit; PerkinElmer, NEL700) for 1 hour and incubated with the anti-DIG-POD Fab fragments (Roche, 1:500) overnight at +4 °C.

The TSA Fluorescence Palette System kit (PerkinElmer, NEL 760) was used to visualize ISH signal.

After ISH signal detection, the samples were washed and IHC was performed as described in Kala et al (2008), except the TNB blocking solution was used for the blocking and antibody dilutions. For nuclear staining, the samples were incubated with DAPI (1:50000 in PBS) for 5 min. Slides were mounted in Mowiol.

***In ovo* electroporation**

Fertilized Dekalb hen eggs were used in all chicken experiments. Eggs were stored at +12 °C until use (for max. 4 days), and placed to the incubator at +37.8 °C to reach desired stages. For most manipulations, 33h incubation time was used (Hamburger-Hamilton stage (HH) 12-14 embryos). DNA solution (final concentration of 1µg/µl, supplied with Fast Green for visualization) was microinjected into the midbrain vesicle. For the electroporation, ECM 830 electroporator and GenePaddles (3x5 mm) electrodes (BTX, cat.no 45-0169) were used. Electroporation conditions were as follows: voltage, 20 mV; pulse length, 20 milliseconds (ms); pulse interval, 500 ms; 10 pulses.

After manipulations, eggs were supplied with 1-2 ml of PBS (containing 0.25% Penicillin, 0.25% Streptomycin, to avoid infections), sealed with tape and placed back to the incubator. The embryos were harvested 24 or 48 hours later and processed for analyses.

Table 3. Transgenic mouse lines used in this study

Transgenic allele	Reference	Publication
Ascl1KO (Mash1null)	(Guillemot et al., 1993)	I, II
Engrailed1-Cre (En1-Cre)	(Kimmel et al., 2000)	I, II, III
Foxg1-Cre	(Hebert and McConnell, 2000)	III
Gata2 flox	Haugas et al., 2010, in press	I, II, III
Gbx2-CreERT2	(Chen et al., 2009)	III
HeltKO (MgntZ)	(Guimera et al., 2006b)	I
R26R	(Soriano, 1999)	III
Shh-Cre	(Harfe et al., 2004)	III

Table 4. PCR genotyping

Transgenic allele	Primers (5' - 3')	expected PCR product(s)
En1-Cre	Cre3: CGT TTT CTG AGC ATA CCT GGA Cre5: AAT CTC CCA CCG TCA GTA CG	Cre^+: 500 bp
Gbx2-CreERT2		
Shh-Cre		
Foxg1-Cre		
Gata2 flox	G2-kond-F: CTT TCC ACC CTC CTT GGA TT G2-kond-R: TTT TTC CCC AAA GTC ACC TG	wt: 490 bp flox: 520 bp
R26R	R1295: GCG AAG AGT TTG TCC TCA ACC R523: GGA GCG GGA GAA ATG GAT ATG R26F2: AAA GTC GCT CTG AGT TGT TAT	wt: 600 bp R26-LacZ: 325 bp
Ascl1KO	Mash1-F: CCA GGA CTC AAT ACG CAG GG Mash1ko-R: GCA GCG CAT CGC CTT CTA TC Mash1wt-R: CTC CGG GAG CAT GTC CCC AA	wt: 600 bp KO: 650 bp

Table 5. ISH probes used in this study.

Mouse probes	**Publication**	**Reference/ Source**
Ascl1	I, II	(Jukkola et al., 2006)
Corin	III	(Ono et al., 2007)
Delta1	II	IMAGE p968BO7112D6
Fev (Pet1)	I	(Jukkola et al., 2006)
Gad1 (Gad67)	I, II, III	(Guimera et al., 2006b)
Gad2	I	(Guimera et al., 2006b)
Gata2	I, III	(Lillevali et al., 2004)
Gata3	I	(Lillevali et al., 2004)
Gbx2	III	(Trokovic et al., 2003)
Helt	I	(Guimera et al., 2006b)
Hes5	II	Gift from I. Thessleff
Isl1	I	gift from V. Pachnis
Lmx1b	I	gift from H. Simon
Ngn1	II	IMAGE p968GO3104D
Ngn2	I, II	(Jukkola et al., 2006)
Nkx2-2	I, II	IMAGE clone 480100
Otx2	I, III	(Acampora et al., 1997)
Pax6	I	gift from P. Gruss
Pitx2	I	(Guimera et al., 2006b)
Pou4f1	I	gift from Siew-Lan Ang
Slc17a6 (Vglut2),	I, II	(Guimera et al., 2006b)
Chicken probes		
cGad1	I	(Cheng et al., 2004)
cGata3	I	(Lillevali et al., 2007)
cNgn2	I	(Matter-Sadzinski et al., 2001)
cSlc17a6	I	(Cheng et al., 2004)

Table 6. Primary antibodies used in this study.

Raised in	Antigen	Publication	Source/ Product number	dilution
guinea pig	Heslike (Helt)	I, II	gift from R. Kageyama	1:500
goat	HA-probe	I	Santa Cruz sc-805-G	1:500
	Olig2	I	Neuromics GT15132	1:200
mouse	BrdU	I, II, III	GE Healthcare RPN20AB	1:400
	HuC/D	I, II	Molecular Probes A21271	1:500
	Lim1/2 (Lhx1)	I, II	Developmental Studies Hybridoma Bank (DSHB) 4F2	1:10
	Mash1 (Ascl1)	I	BD Biosciences 556604,	1:200
	Nkx2-2	I, II	DSHB 74.5A5	1:250
	Nkx6-1	I, II	DSHB F55A10	1:500
	p27	II	BD Transduction labs 610241	1:800
	Pax6	I, II	DSHB PAX6	1:100
	Pou4f1	I, II	Santa Cruz sc-8429	1:400
	TH	III	Millipore MAB318	1:300
rabbit	5-HT	I	Immunostar 20080	1:5000
	β-gal	III	MP Biomedicals 55976	1:1000
	Active caspase3	I, II	R&D Systems AF835	1:500
	CyclinD1	II	Lab-vision RM-9104-SO	1:400
	Gata2	I, II	Santa Cruz sc-9008	1:250
	Lmx1a	III	gift from Michael German	1:750
	p27	II	Thermo Scientific Rb-006-P0	1:100
	p57	I, II	NeoMarkers RB-1637-P0	1:500
	phospho-histone H3	I	Upstate 06-570	1:500
	Sox2	I, II	Millipore AB5603	1:500
	TH	I, III	Millipore AB152	1:500

4. RESULTS

4.1. The expression of Gata2 and Gata3 in the midbrain (I)

It was previously reported that Gata2 and Gata3 are expressed in the midbrain-r1 region in the mammalian CNS. However, the cell type specificity of these TFs had not been unambiguously demonstrated. To identify the neurotransmitter phenotype of *Gata2/3*-expressing cells in midbrain, we compared *Gata2* and *Gata3* expression to cell type specific markers during the development of mouse midbrain and r1. These comparisons revealed that the expression of Gata2 and Gata3 mRNA and protein fully coincide with the expression of GABAergic neuron marker *Gad1* and is excluded from the cell groups expressing a glutamatergic neuron marker *Vglut2* (vesicular glutamate transporter-2). As in ventral spinal cord and r1, *Gata2* expression precedes *Gata3* in the midbrain. We detected *Gata2* transcripts mostly in the intermediate zone of neuroepithelium (the cells at the border of VZ and MZ as determined by the expression of stem cell marker Sox2 and neuronal protein HuC/D, respectively) and in scattered pattern also in the ventricular zone, where proliferative neuronal precursors reside. *Gata3* expression in midbrain seems to follow *Gata2* and was only detected in differentiating *Gad1*$^{+}$ GABAergic neurons in the marginal zone.

Temporally, the expression of *Gata2* and *Gata3* starts from the ventrolateral midbrain, where we detected strong expression of both genes as well as *Gad1* at E11.5. By E12.5, Gata2 and Gata3 expression is detectable also in the dorsal midbrain (m1-m2 domains), consistent with the later onset of GABAergic neurogenesis in dorsal as compared to ventral midbrain (Fig. 1 in I).

In conclusion, we demonstrated that the expression of Gata2 and Gata3 both spatially and temporally correlates with the GABAergic neurogenesis. This expression pattern of Gata2 and Gata3 suggests that these factors may be involved in GABAergic neuron development.

4.2. Patterning of the midbrain neuroepithelium (I)

Recently, a useful DV gene expression map was published by Nakatani et al (2007). To identify the precise positioning of Gata2 and Gata3 in this map, we performed similar analysis of DV patterning genes, bHLH TFs and GATA TFs in midbrain progenitor cells and differentiating neurons at E11.5-E12.5.

Our expression mapping results are consistent to the model proposed by Nakatani et al., but provide further detail (Figure 9). We demonstrated that Gata2 and Gata3 are expressed in the differentiating cells of all domains associated with GABAergic neurogenesis, m1-m5. The progenitor cells of the same regions are characterized by Ascl1 and Helt expression. In addition, we identified a previously unrecognized heterogeneity in m4 domain, which apparently consists of *Gad1*- and Gata2/3-positive domain (m4-d) and *Gad1*- negative, Pax6-positive (m4-v) domain. The m4-v is active in glutamatergic neurogenesis, as identified by *Vglut2* expression in Pax6^{+} cells (Supp.Fig. S1 in I), but unlike the rest of the glutamatergic cells in midbrain, m4-v cells do not express Pou4f1.

In summary, the spatial mapping demonstrated that the expression of Gata2 and Gata3 follows the domain boundaries established in earlier stages of development by combinatorial TF expression. Our results also refined the fate map of the lateral midbrain (m4).

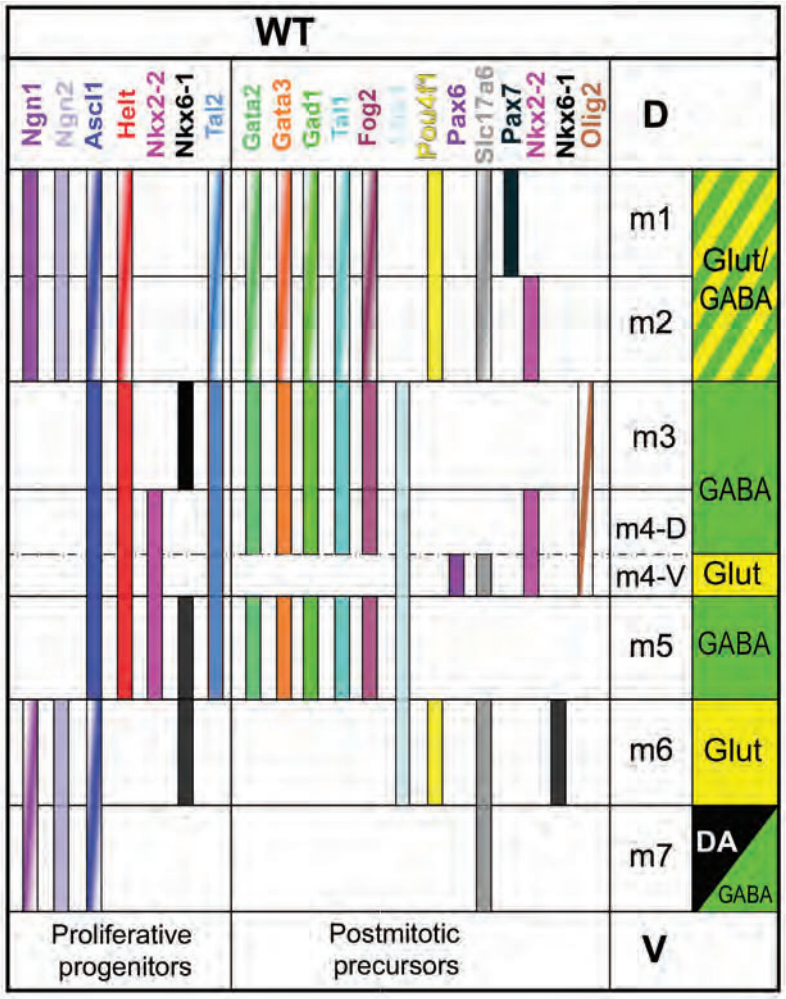

Figure 9. The expression of HD patterning genes, bHLH TFs, GATA factors and neuronal subtype specific genes in the proliferative ventricular zone progenitors and marginal postmitotic neuron precursors in midbrain DV domains (m1-m7). Expanded from Fig. 8 in (I).

4.3. Gata2 and transcriptional regulators of GABAergic fate in midbrain (I, II)

Next, we aimed to identify the upstream molecular pathway regulating Gata2 expression in the midbrain. Considering the specificity of *Gata2* to the domains of GABAergic neurogenesis, which in turn correlate with the VZ domains expressing bHLH TFs involved in midbrain GABAergic neuron development (i.e. Ascl1 and Helt), we

analyzed Gata2 protein expression in respect to these transcription factors. Consistent with the mRNA expression patterns, all three proteins were coexpressed in the VZ progenitors. However, the immunohistochemistry (IHC) revealed notable differences in their expression kinetics. We found that both Helt and Gata2 are predominantly localized in Ascl1 expressing cells, while only few cells coexpress Helt and Gata2. In addition, the nuclei of Ascl1$^+$ cells are uniformly distributed along the apical-basal width of the VZ, while the nuclei of Helt-expressing cells are predominantly found at the apical side and Gata2 at the opposite, basal side. As follows from the INM dynamics during cell cycle progression, Helt would be expressed either in mitotic or newly born neuronal progenitors/precursors. In contrast, Gata2 could be expressed either in S-phase cells or postmitotic neuronal precursors that are leaving VZ to differentiate in more basal, marginal zone. We addressed this point by analyzing the proliferation and differentiation status of Gata2$^+$ cells. Gata2$^+$ cells did not incorporate S-phase marker BrdU after a short BrdU pulse, arguing against the expression of Gata2 in cycling neuronal progenitors. Instead, we detected coexpression of neuronal differentiation marker HuC/D in many Gata2$^+$ cells, supporting the hypothesis of post-mitotic onset of Gata2 expression (Fig. 2 in I).

In summary, these results showed that Ascl1 and Helt may be expressed in the proliferative progenitors, whereas Gata2 expression can be detected only after the cell has become postmitotic, committed GABAergic precursor (Figure 10).

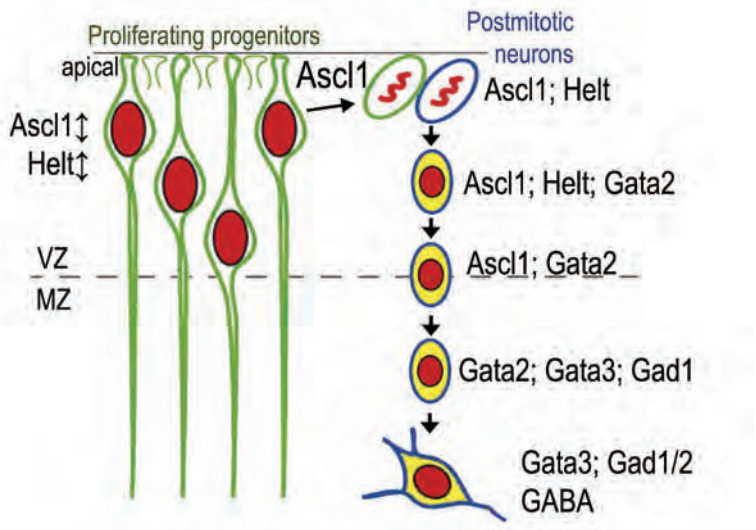

Figure 10. Schematic representation of Ascl1, Helt and Gata2 expression kinetics during the GABAergic neurogenesis in the midbrain. The proliferative VZ precursors express varying levels of Ascl1 and Helt. Stabilization of Ascl1 expression triggers neurogenic cell cycle exit. Helt and Ascl1 are coexpressed in early GABAergic precursors. As the precursors move out of the VZ towars MZ, Helt is downregulated and Gata2 upregulated. In the VZ-MZ border, GABAergic progenitors express Ascl1 and Gata2. During differentiation in MZ, Gata3 and GABAergic function specific genes *Gad1/2* are expressed. Modified from Zhong and Chia, 2008.

To study the functional relevance of the discovered relationships, we analyzed Gata2 expression in the mouse embryos lacking Ascl1 (Ascl1KO) or Helt (HeltKO). As *Gata2* was absent in most of the midbrain neuroepithelium in HeltKO embryos, we concluded that Helt is required for Gata2 expression in midbrain, excluding the most ventral GABAergic domain, m5. On the other hand, in *Ascl1* mutant midbrains, *Gata2* expression is initiated in the ventrolateral midbrain (m3-5), but missing in dorsal regions (m1-2), demonstrating spatially differential requirement of Ascl1 for *Gata2* expression (Fig. 3 in I; Fig. 4 in II). At least in m3-5, Ascl1 may act as a general regulator of neurogenic cell cycle exit and Helt may primarily regulate the Gata2 expression. In the dorsal midbrain, the presence of both Ascl1 and Helt appears to be required to activate Gata2 expression. However, it is possible that in the absence of Ascl1, dorsal GABAergic progenitors are blocked at cell cycle exit because of the lateral inhibition received from neighbouring glutamatergic progenitors. Thus, the Ascl1 mutant progenitors may never reach the stage where *Gata2* would be expressed.

4.4. The function of Gata2 in GABAergic neuron development (I, III)

We next studied the role of Gata2 during the development of diencephalic, midbrain and r1 GABAergic neurons using conditional deletion of floxed Gata2 allele (Gata2 flox; Maarja Haugas, Kersti Lilleväli, Marjo Salminen) with either En1-Cre (midbrain and r1; Kimmel et al., 2000) or Foxg1-Cre (whole brain neuroepithelium; Hebert and McConnell, 2000).

4.4.1. Midbrain (I)

4.4.1a. Gata2 loss-of-function

- **GABAergic neurons are lost in the absence of Gata2**

In the En1-Cre/+; Gata2 flox/flox (Gata2cko) embryos, we detected no expression of GABAergic neuron markers *Gad1*, *Gad2* and *Gata3* in the midbrain MZ at E11.5-13.5, demonstrating a complete loss of postmitotic GABAergic neurons. At the same time, expression of genes associated with progenitor domain patterning and proneural function, such as Ascl1, Ngn2, Helt, Nkx2-2 and Nkx6-1 in the VZ, were unaffected (Fig.4 A-P, and Supp.Fig S3 in I).

We analyzed the Gata2cko phenotype also in the perinatal (E18.5) brains, where midbrain morphogenesis is more complete. The loss of GABAergic neurons persisted also at the later stages, as the loss of *Gata3*, *Gad1* and mature GABAergic neuron marker *Pitx2* could not be detected in these Gata2cko brains (Supp. Fig. S5 in I).

These results correlate well with the onset of *Gata2* expression in early postmitotic stage and suggest that Gata2 function is crucial for the commitment to GABAergic fate.

- **Apoptosis and progenitor proliferation are unaltered in the Gata2cko**

Next, we investigated the possible mechanisms behind the apparent loss of GABAergic neurons in the Gata2cko. Despite the substantial defect in neurogenesis, the overall morphology of Gata2cko embryos is indistinguishable from the wild-type (wt)

littermates, making cell death an unlikely explanation for the loss of GABAergic neurons. Consistently, the number of apoptotic cells was not increased in the Gata2cko midbrains at E11.5 (Supp. Fig. S4 in I). Gata2 has been reported to drive cell cycle exit in chicken spinal cord (El Wakil et al., 2006). In the midbrain, Gata2 is expressed in the postmitotic neuronal precursors already in the VZ, and thus might regulate cell cycle exit. However, we detected no alteration in the numbers of mitotic cells and only a minor change in BrdU incorporation was observed in Gata2cko versus wt m3-5 neuroepithelium. Also, we detected no changes in the thickness of the $Sox2^+$ progenitor layer and HuC/D^+ MZ in the Gata2cko (Supp. Fig. S4 in I), and the VZ progenitor patterning was unaltered, as mentioned above.

These results showed that the cell death or alterations in the progenitor proliferation or neurogenic cell cycle exit could not explain the loss of GABAergic neurons in the Gata2cko.

- **GABAergic-to-glutamatergic fate transformation in the absence of Gata2**

We then considered the possibility that the loss of the GABAergic neuron markers would indicate an incomplete differentiation of Gata2-mutant precursor cells or a re-specification into another phenotype.

Supporting the latter hypothesis, we observed a complementary upregulation of glutamatergic neuron marker *Vglut2* in the prospective GABAergic domains in the Gata2cko midbrains (Fig. 4A-D in I). Thus, Gata2 is acting as a post-mitotic selector gene for inhibitory as opposed to an excitatory fate, and in the absence of Gata2 all midbrain-derived precursors fail to activate GABAergic pathway and switch to the glutamatergic, *Vglut2*$^+$ phenotype instead. The genetic diversity already observed in the progenitors domains in the midbrain VZ, is here well illustrated also within the midbrain GABAergic precursors. More specifically, we found that in the absence of Gata2, the cells not merely switch on the general glutamatergic neuron marker *Vglut2*, but specifically acquire the expression of markers characteristic for the glutamatergic precursor subtypes in their adjacent compartment. For example, in wt brains, the GABAergic m5 domain cells are $Lhx1^+$ while their neighbouring glutamatergic cells in m6 compartment are $Nkx6\text{-}1^+$. In the Gata2cko, the post-mitotic precursors in m5 domain become $Nkx6\text{-}1^+$, and lose the Lhx1 expression, resembling the m6 glutamatergic cells. Similarly, the m4-v glutamatergic progenitor marker Pax6 expands also to the cells of m4-d (Fig. 4Q-X in I). In the most dorsal GABAergic domain (m3), the precursors switch on the expression of Pou4f1, a marker of dorsal midbrain glutamatergic neurons (Fig. 4G,H in I).

Thus, in the absence of Gata2, prospective midbrain GABAergic precursors undergo a fate transformation and switch to the glutamatergic differentiation programme.

- **GABAergic neurons associated with vMB DA nuclei do not require Gata2**

Perhaps the most striking aspect of Gata2cko phenotype was revealed in our analysis of Gata2cko midbrains at prenatal stage (E18.5), where the morphology resembles adult organization. Despite the complete lack of GABAergic neuron production from the midbrain neuroepithelium at E11.5-E13.5, and the GABA-to-glutamatergic fate transformation in all other midbrain GABAergic nuclei at E17.5-E18.5, we detected apparently normal numbers of *Gata3* and *Gad1* expressing GABAergic neurons in the SNpr and VTA of the E18.5 Gata2cko brain (Fig. 7 in I).

Considering the crucial role of Gata2 for GABAergic neuron differentiation in midbrain, this observation strongly indicated a distinct origin and/or developmental mechanisms for the VTA-SNpr GABAergic neurons.

4.4.1b. Gata2 gain-of-function

- **Gata2 induces glutamatergic-to-GABAergic fate transformation**

The analyses of Gata2cko phenotype revealed strict requirement of Gata2 for GABAergic neuron development in the mouse midbrain. When ectopically introduced to chick neural tube, Gata2 induces cells to acquire V2b interneuron phenotype in the ventral spinal cord (Karunaratne et al., 2002) and serotonergic neuron fate in the anterior hindbrain (Craven et al., 2004), in both cases at the expense of the neighbouring cell fates. We decided to test if Gata2 can similarly trigger GABAergic neuron development in the midbrain.

We first compared the expression of GABA- and glutamatergic neuron markers in the chick and mouse midbrain. Similar to mouse, we found *Helt* expression in the VZ progenitors giving rise to Gata2, Gata3, Lhx1/2 and Gad1 -positive GABAergic neuron precursors in the chick midbrain. At the same time, *Ngn2* was expressed on the basal side of glutamatergic progenitor domain identified by *Vglut2* expression in the MZ. Notably, we also observed a *Gad1* negative domain in the ventrolateral chicken midbrain, a likely counterpart of mouse m4-v domain. As GABAergic development seems to occur in similar fashion in the mouse and chick midbrain, we employed the chick model to demonstrate effects of *Gata2* overexpression to the neurogenesis in the midbrain. Using *in ovo* electroporation to deliver *Gata2* expression vector in chicken dorsal midbrain, we showed that Gata2 can induce the expression of *Gata3*, Lhx1/2 and *Gad1*, genes associated with the GABAergic differentiation pathway, in the targeted areas. In the same areas, Vglut2 expression seemed to be downregulated (Fig. 5 in I).

Thus, the Gata2 gain-of-function analyses demonstrated that Gata2 is not only necessary, but also sufficient to switch on the GABAergic neuron differentiation in midbrain, and suggested that the induced GABAergic neurons are specified in expense of glutamatergic neurons.

4.4.2. Diencephalon (I, III)

In addition to the midbrain, Gata2 is strongly expressed also in the diencephalic structure dorsal thalamus at the time and place of GABAergic neurogenesis. However, the possible function of Gata2 in the diencephalon could not be addressed in the Gata2cko, since En1-driven Cre is not active in that region (Supp. Fig. 2 in I). Using Foxg1-Cre mediated recombination, we could address the function of Gata2 also in the dorsal thalamus.

The analysis of Foxg1-Cre; Gata2 flox/flox embryos revealed the loss of GABAergic markers *Gad1* and *Gata3* in the Gata2-deficient diencephalon (Fig. 1 in III). The switch into a *Vglut2*$^{+}$ glutamatergic phenotype was also observed in the respective domains (S. Virolainen, K. Kala, unpublished results).

These results demonstrated that similar to midbrain, Gata2 is acting as a neuronal subtype selection determinant in the diencephalic GABAergic neurons.

4.4.3. Rhombomere 1 (I)

4.4.3a. GABAergic neurons

Similar to midbrain and dorsal thalamus, we detected Gata2 and Gata3 expression also in the domains of GABAergic neuron production in r1.

However, in contrast to the conspicuous phenotype in midbrain and diencephalon, the expression of *Gata3*, *Gad1* and *Pitx2* appeared completely unaffected in the r1 GABAergic neurons in the Gata2cko (Fig. 6 and Supp. Fig. S5 in I). Thus, Gata2 is essential for GABAergic neurogenesis in the midbrain and diencephalon but dispensable for that matter in the r1. It is possible that GATA TFs are not involved in the GABAergic neurogenesis in the hindbrain at all, but this is perhaps unlikely since both *Gata2* and *Gata3* are strongly expressed in the respective domains in r1. Alternatively, Gata3 might be able to compensate for Gata2 function in the r1, but not in the midbrain.

This result exemplifies once more the diverse mechanisms leading to the generation of similar cell type, the GABAergic neuron, in different (yet adjacent) brain compartments.

4.4.3b. Serotonergic neurons

Gata2 has been reported as a crucial determinant of the r1 serotonergic neuron fate (Craven et al., 2004). However the previous studies have been limited due to the lethality of ubiquitous Gata2 inactivation at E9.5 and in the study by Craven et al (2004), the serotonergic neuron development has been followed using in vitro cultures of the Gata2 mutant tissue. We could test these findings in a normal biological context of developing embryo, as Gata2cko mutation is viable.

Consistent to the results of Craven et al (2004), we were unable to detect the expression of any serotonergic neuron marker, including Lmx1b, Pet1 or 5-HT in the Gata2cko r1. In addition, we detected no expression of Gata3 in the Gata2cko serotonergic compartment, while it was unaffected in the GABAergic neurons (Fig. 6 in I). Therefore, in contrast to Craven et al (2004), we find that Gata2 is required for Gata3 expression in the r1 serotonergic neurons.

In conclusion, we confirmed that Gata2 is required for the serotonergic neuron development in r1, and showed that in addition to other serotonergic neuron specific genes, Gata2 apparently also regulates the expression of Gata3 in these cells.

4.5. The function of Ascl1 in GABAergic neuron development (II)

Another TF associated with the domains of GABAergic neurogenesis in the mouse midbrain is proneural bHLH protein Ascl1 (Fig. 1 in II). Failure of GABAergic neuron development has been reported in Ascl1KO (Miyoshi et al., 2004). However, only an early stage of GABAergic neuron development was analyzed in this study. Diversity in Ascl1 function has been observed in the mouse forebrain, where Ascl1 is required for GABAergic neuron production in MGE, while in LGE this requirement is not absolute (Casarosa et al., 1999). We have studied the requirement of Ascl1 in the midbrain and r1 GABAergic neurons, providing evidence of complex requirements for Ascl1 also in this region.

4.5.1. Dorsal midbrain: Ascl1 required for GABAergic neurogenesis

Our analyses of the Ascl1KO phenotype show strict requirement for Ascl1 during the GABAergic neuron development in the dorsal midbrain (m1-2 domains). In Ascl1KO, *Gata2* and *Gad1* expression is absent from this region at E11.5-E13.5, when active neurogenesis takes place (Fig. 3 in II). The defective neurogenesis if followed by a complete loss of GABAergic neurons from the dorsal MB derivatives, SC and dPAG in the E16.5 embryos (Fig. 2 in II). These observations indicate that Ascl1 function is required for the GABAergic neurogenesis in dorsal midbrain, as has been proposed previously (Miyoshi et al., 2004). Also, we observed no differences in Helt expression in the dorsal midbrain. During normal development, Helt expression in m1-2 is initiated slightly later than in ventral domains (m3-5), and both in the wt and Ascl1KO, the characteristic salt-and-pepper pattern of Helt$^+$ cells was obvious (Supp. Fig. 1 in II).

4.5.2. Ventral midbrain: Ascl1 promotes the onset of GABAergic neurogenesis

In contrast to m1-m2, we find Ascl1 not required for the differentiation of GABAergic neurons in more ventral midbrain nuclei, but rather for the timing of neurogenesis. The differentiation of both Gata2$^+$ GABAergic and Pax6$^+$ glutamatergic precursors was markedly delayed in the Ascl1KO as compared to the wt midbrains. At the same time, increased thickness of the Sox2$^+$ progenitor cell layer indicated a general defect in the cell cycle exit. However, we observed a recovery in the neurogenesis by E13.5 (Fig. 4 in II). Consistently, GABAergic neurons were not lost from prenatal ventral midbrain derivatives mRF and vlPAG (Fig. 2 in II). Thus, unlike in dorsal midbrain, ventrolateral midbrain GABAergic neurons can be produced in absence of Ascl1, but their differentiation seems to be initiated at a later time point. Despite the delayed onset of their differentiation, the m3-5 progenitors seem to be depleted at the same time in Ascl1KO and wt, as the thickness of progenitor cell layer (Sox2$^+$) was similar in the control and Ascl1-mutant midbrains at E13.5 (Fig. 4 in II). This means that time period permissive of GABAergic neurogenesis might be significantly shortened due to the initial delay in differentiation, supported by the apparent reduction in the numbers of GABAergic neurons found in the ventral midbrain nuclei later in development.

4.5.2a. Loss of lateral inhibition

As a proneural TF, Ascl1 is most probably involved in the lateral inhibition, via the activation of Delta and triggering Hes expression in the neighbouring cells. Therefore, we asked if alterations in Notch pathway targets and/or cell cycle regulator expression could contribute to the neurogenesis defects observed in Ascl1KO midbrains. Indeed, the absence of Ascl1 results in downregulation of *Dll1* and *Hes5* in the m3-5 at E11.5. In addition, we demonstrated upregulation of Helt in the E11.5 Ascl1KO ventrolateral midbrain, where nearly all VZ cells appeared to express Helt protein in contrast to the salt-and-pepper pattern observed in the wt m3-5, or both control and Ascl1KO dorsal midbrain. Strikingly however, we observed the recovery of normal Dll1, Hes5 and Helt expression patterns together with the neurogenesis by E13.5. It is possible that another proneural factor is expressed in the absence of Ascl1 in the ventrolateral midbrain. One candidate would be Ngn1/2, which is expressed in mutually exclusive domains in respect to Ascl1 in the midbrain. However, we did not observe any expansion of *Ngn1/2* to the

Ascl1-deficient areas in the m3-5 or upregulation of its expression in the m1-2 (Fig. 5 in II). The upregulation of Ngn1/2 would perhaps also be unexpected due to the maintained Helt expression in the Ascl1KO, as Helt acts as a repressor of Ngn-s (Nakatani et al., 2007). To date, the mechanism behind the re-activation of Dll1 and Hes5 in the Ascl1 deficient m3-5 remains to be identified.

4.5.2b. Aberrant cell cycle exit in the absence of Ascl1

We also analyzed the expression of cell cycle regulators in the m3-5 domains of Ascl1KO. Cyclin-dependent kinase inhibitors p57 and p27 are associated with the postmitotic decision in the cycling progenitors. Both of these proteins were clearly expressed and locally even upregulated in the Ascl1KO at E11.5, showing that even at the time of suppressed neurogenesis, Ascl1 mutant cells are attempting cell cycle withdrawal. The increase in p27/p57^{+} cell numbers might indicate a defect in the progression from that particular step, for example due to incomplete instructions for differentiation. How and if Ascl1 regulates the onset of differentiation gene expression is unclear. Rather, it is likely that in the absence of Ascl1 progenitor cells can not readily acquire competence for differentiation. In addition, we noticed clear differences in how ventrolateral midbrain VZ cells tolerate the loss of Ascl1. For example, the m4 domain cells seem to be the earliest to recover from the differentiation arrest, since Gata2 and p27 expression is first detected in this domain, also HuC/D seems to be expressed earlier (Fig. 6 in II).

Altogether, our analyses of the Ascl1KO phenotype in ventrolateral midbrain demonstrated diversity in the cellular tolerance to the loss of Ascl1. In contrast to its requirement for GABAergic differentiation in dorsal midbrain, the main function of Ascl1 in the ventral midbrain seems to be to regulate the timing of GABAergic neurogenesis.

4.5.3. VTA and SNpr: Ascl1 is dispensable for GABAergic neuron development

We next looked more closely at the most ventral midbrain GABAergic neurons in the SNpr and VTA, which were unaffected by the loss of Gata2. In the Ascl1-mutant prenatal midbrains, *Gad1*$^{+}$ GABAergic neurons in the SNpr and VTA appeared normally present. Unlike the other GABAergic nuclei, mRF and vlPAG, we did not notice reduction in the intensity of *Gad1* expression in the SNpr or VTA, indicating that there is no reduction in the cell numbers in these nuclei (Fig. 2 in II).

Therefore, similar to Gata2, Ascl1 appears to be dispensable for the development of GABAergic neurons associated with midbrain DA nuclei.

4.5.4. Rhombomere 1: Ascl1 is dispensable for GABAergic neuron development

Ascl1 has also been shown to be expressed in the hindbrain. We confirmed this, showing the expression of Ascl1 in the progenitor domains producing GABAergic neurons in r1 and also in more caudal rhomobomeres. However, we did not notice defects in the *Gad1* expression in the r1 of Ascl1KO at E13.5 or E16.5. In this aspect, Ascl1KO is again similar to Gata2cko, where r1 GABAergic neurons are also normally present (Fig. 3 in II).

In summary, our analyses showed that Ascl1 is involved in the development of the GABAergic neurons in the midbrain, but not in the r1.

4.6. Origin of VTA-SNpr GABAergic neurons (III)

As the GABAergic neurons in SNpr and VTA are not affected by the loss of Gata2 or Ascl1, we reasoned that these cells may originate from adjacent brain regions that also remain unaffected in the Gata2cko or the Ascl1KO. These regions include the diencephalon, which remained unaffected in the Gata2cko, and r1, where GABAergic neurogenesis does not require Gata2 or Ascl1 (I,II). Considering these possibilities, we underwent detailed fate-mapping to identify the spatiotemporal origin of the SNpr and VTA GABAergic neurons (vMB GABAn).

4.6.1. Normal development of vMB GABAn despite the lack of GABAergic neurogenesis in dorsal thalamus

As described in 4.4.2., we designed a general CNS deletion of Gata2 using the Foxg1-Cre, which is active throughout the developing neuroepithelia. In the Foxg1-Cre; Gata2flox/flox brains, GABAergic neurons were missing in the diencephalic derivative dorsal thalamus and, consistent with the Gata2cko, also in midbrain, but unaffected in the r1. Importantly, we also observed normal development of vMB GABAn in these mutant brains (Fig. 1 in III).

This data suggests that the origin of vMB GABAn in diencephalon is less likely than origin in r1.

4.6.2. Genetic fate mapping by Cre-recombinase

We utilized Cre recombinase expressing mouse lines to label cells born in distinct areas in R26R Cre-reporter mouse, and followed the beta-galactosidase (β-gal) expression in the vMB GABAergic and dopaminergic cells. With En1-Cre, we could identify cells originating from midbrain and r1, and vMB GABA- as well as DA neurons were efficiently labelled in En1-Cre; R26R brains (Fig. 2 in III). Therefore, vMB GABAn originate from the MB-r1 area.

To separate cells originating from midbrain and r1, we used r1-specific Cre expressed in the $Gbx2^{+}$ domain (Gbx2-CreERT2, Chen et al., 2009). CreERT2 protein expressed in this mouse line can be activated by tamoxifen (tx) administration. For our analyses, we induced Cre activity at E8.5, a stage where clear Otx2 and Gbx2 expression border at MHB is established (Supp.Fig, S1 in III). After tx administration, recombinase activity of CreERT2 persists for 36-48h (Hayashi and McMahon, 2002), allowing us to follow the descendants of cells expressing Gbx2 at E8.5-10.0 (10.5). Prominent β-gal expression in the vMB was conspicuous in the E18.5 Gbx2-CreERT2; R26R brains. Furthermore, this labelling was predominantly restricted to GABAergic and not DA neurons (Fig. 3 in III).

However, we observed that in addition to r1, some midbrain floor plate cells are also labelled by Gbx2-CreERT2 at E9.5 (Fig. 4 in III). As the midbrain floor plate has been reported to be neurogenic (Joksimovic et al., 2009b), the labelled cells in SN-VTA of E18.5 brains could be derived either from this population, or from r1. To distinguish between these options, we used Shh-Cre that is active in only the floor plate cells in r1 (which are non-neurogenic), but in the midbrain its activity extends to adjacent basal midbrain cells in addition (Joksimovic et al., 2009b). Shh-Cre efficiently labelled DA cells in the ventral midbrain. In contrast, most of the GABAergic cells in the same region were $\beta\text{-gal}^{-}$ (Fig. 5 in III).

Thus, we concluded that SNpr and VTA GABAergic cells primarily do not originate in the ventral midbrain neuroepithelium, but instead are derived in r1, excluding its floor plate.

4.6.3. Birth-dating of midbrain GABAergic neurons by BrdU labelling

Having identified the origin of vMB GABAn in the r1, we wanted to follow the timing of their appearance. Using BrdU-labelling over long (4-7 days prior to birth) and short time periods (18 hours saturation starting at different developmental stages between E9.5 and E13.5), we determined the time point of the final mitoses and the stage of the peak in neurogenic division for the *Gad1*$^+$ cells in different midbrain areas: r1 derived SNpr, ventral midbrain derived mRF and dorsal midbrain derived SC.

The birth-dating analyses showed that SNpr GABAergic neurons are generated during E11.5-E13.5, with a peak at E11.5. Similar results of the time or origin of SN and VTA cells have been obtained in rat, although this study did not distinguish neuronal subtypes (Altman and Bayer, 1981). The timing of vMB GABAn production appears slightly different from the *Gad1*$^+$ cells in other midbrain nuclei included in our analyses. In short, the ventral midbrain derived mRF neurons seem to be produced slightly earlier, while dorsal midbrain neurons in a later and longer-lasting wave of neurogenesis. The neurogenesis concerning SNpr cells is completed by E13.5, a day later than the ventrolateral midbrain-derived mRF, but earlier than in dorsal midbrain, where some GABAergic cells still seem to be generated as late as E14.5 (Fig. 6A-B in III).

Thus, the GABAergic neurons in different midbrain nuclei, including the SNpr-VTA, are produced with distinct kinetics.

4.6.4. GABAergic neuron migration from r1 to ventral midbrain

Our birth-dating analyses demonstrated that the SNpr GABAergic neurons are produced during E11.5-E13.5. At these stages, no *Gad1*$^+$ GABAergic neurons could be detected in the Gata2cko midbrains, or in the VTA or SN area, defined as the immediate proximity of TH$^+$ cells, in the wt ventral midbrains. We again utilized Gbx2-CreERT2; R26R mice and were able to observe the appearance of β-gal$^+$ and *Gad1*$^+$ cells in the midbrain neuroepithelium. In these embryos, we detected the first β-gal$^+$ cells in vMB at E14.5. More β-gal$^+$ cells are present in midbrain at E15.5, by which stage we could also detect first *Gad1*$^+$ cells in the area associated with TH$^+$ cells both in wt and Gata2cko. By E16.5, GABAergic neurons clearly populate SNpr area and these cells were also labelled by Gbx2-CreERT2 (Fig. 6 C-R in III).

Taken together with the fate-mapping and birth-dating data, the timing of GABAergic neuron appearance suggests that r1-derived vMB GABAn relocate into their final positions in VTA-SN area as post-mitotic precursors.

4.7. Gata2 binding partners (unpublished)

We next aimed to identify potential targets of Gata2 in the mouse midbrain. For this, we chose to compare the global gene expression in the wt and Gata2cko mouse embryos using a cDNA microarray.

Overall, the results of the microarray analysis correlated well with Gata2cko phenotype we have described. In addition, we depicted several new candidates possibly involved in Gata2-mediated specification of GABAergic fate. For example, GATA-associated proteins like bHLH TFs Tal1 and Tal2, as well as FOG family members Fog1 and Fog2 were represented among the genes downregulated in Gata2cko. These TFs facilitate and modulate the function of GATA proteins. We have confirmed the expression of Tal1, Tal2 and Fog2 in the regions producing GABAergic neurons in the mouse midbrain, and verified their downregulation by ISH analysis of Gata2cko embryos (Figure 11).

Consistently, the regulation of Tal1 midbrain enhancer by Gata2 (and Tal1 itself) has been shown in the developing embryo (Ogilvy et al., 2007).

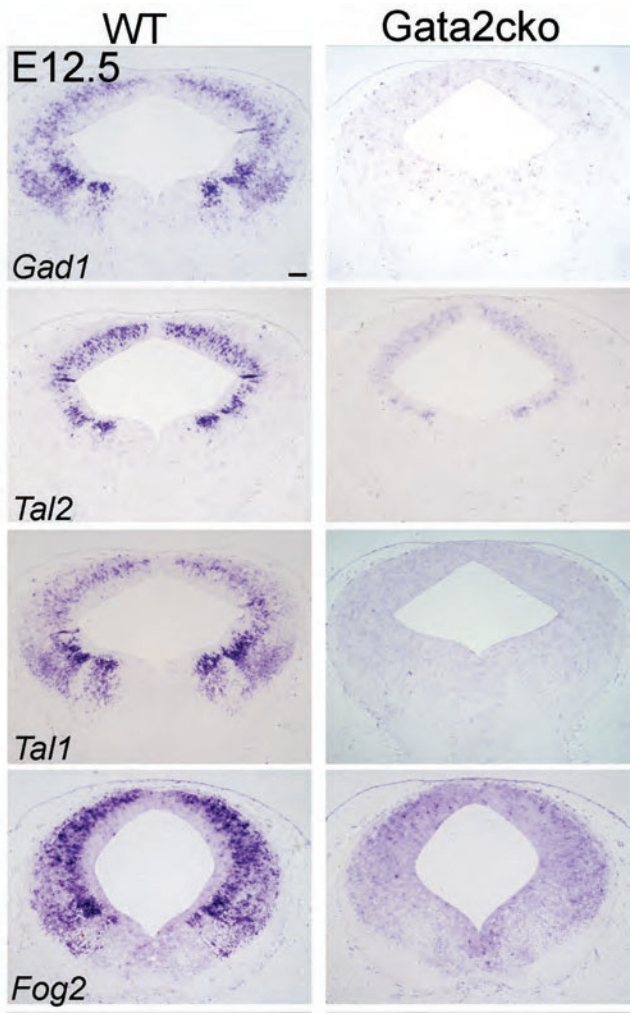

Figure 11. ISH analysis of Gad1, Tal2, Tal1 and Fog2 expression in coronal sections of wt and Gata2cko midbrain at E12.5.

5. DISCUSSION

5.1. The expression of Gata2 and Gata3 in midbrain

Nardelli et al (1999) suggested *Gata2* and *Gata3* are expressed in the motor neurons of oculomotor complex in mouse ventral midbrain, probably because of the proximity of *Gata2*$^+$ cells to the respective arc in chicken midbrain (Agarwala et al., 2001), and the motor neuron phenotype observed in *Gata2*-mutant hindbrain. However, no co-labelling with motor neuron markers were demonstrated in the mouse midbrain, but *Isl1*,*Gata2* double labelled cells were shown only in the hindbrain (Nardelli et al., 1999). In the more caudal regions of CNS like the spinal cord, the Gata2/3 positive cells constitute inhibitory GABA- or glycinergic interneurons (Lundfald et al., 2007).

We have identified the neurotransmitter identity of Gata2/3 expressing cells in the midbrain, showing that these TFs are specifically expressed in the developing GABAergic neurons. In our studies, we have used the expression of GABA synthesizing enzymes, Gad1 and Gad2, to identify GABAergic identity. In addition, we find that Gata3 is also a reliable marker of GABAergic neurons in midbrain. Recently, the coexpression of Gata3 and GABA has also been demonstrated (Joksimovic et al., 2009a).

In addition to the subtype identity of Gata2$^+$ cells, we showed that Gata2 expression is restricted to the early post-mitotic precursors that are exiting the VZ and starting to differentiate. Gata3 expression is regulated by Gata2 and can only be detected in the differentiating GABAergic neurons in the MZ. Similar expression dynamics of Gata2 and Gata3 have been shown elsewhere in the CNS, including the hindbrain serotonergic neurons (Craven et al., 2004, I) and ventral spinal cord precursors (Nardelli et al., 1999; Zhou et al., 2000).

5.2. The expression dynamics of Ascl1, Helt and Gata2

Our comparisons of the Ascl1, Helt and Gata2 protein expression revealed substantial differences in their expression dynamics in the VZ. The changes in these expression profiles likely reflect the different proliferation or/and differentiation status of the VZ cells. The BrdU incorporation analyses have shown that Ascl1 and Helt are both expressed in the proliferative cells (II; Miyoshi et al., 2004), while Gata2 is expressed only post-mitotically (I). Ascl1$^+$ and Helt$^+$ cells are present in salt-and-pepper pattern in the VZ, which might indicate the oscillatory nature of their expression (Kageyama et al., 2008). In addition, Helt expression seems to be cell cycle dependent, as most of the Helt$^+$ cells localize near the apical surface of VZ.

Oscillatory and cell cycle dependent expression of Notch pathway components Delta and Hes1 has recently been observed in cortical neuronal progenitors (Shimojo et al., 2008). In addition, oscillations in the expression of proneural gene Ngn2 have been observed in the cycling neuronal progenitors. Phenomenally, the Hes1 and Ngn2 appear to oscillate in inverse phase (Shimojo et al., 2008). The oscillations in proneural gene levels can be driven by the oscillatory expression of Hes proteins in progenitor cells (Hirata et al., 2002). Indeed, at high levels, Hes1 represses the expression of proneural factors and Notch ligand Delta1 (Shimojo et al., 2008). At low levels of Hes1, proneural genes are derepressed and thus can reactivate Notch signalling via lateral inhibition,

providing a feedback loop maintaining the oscillatory nature of Hes1 gene expression. In this system, oscillatory proneural gene expression would first need to be stabilized in order to drive neurogenic cell cycle exit (Kageyama et al., 2009; Shimojo et al., 2008). Similar to Ngn2 in forebrain, we think the stabilized Ascl1 expression could be associated with the earliest stage of GABAergic neuron development in midbrain, possibly denoting the earliest time point of neurogenic decision, and persist until the exit from VZ. Helt seems to be coexpressed with Ascl1 in the apical neurogenic progenitors and more readily downregulated at the onset of differentiation pathway. As Helt acts as a transcriptional repressor (Miyoshi et al., 2004) and may negatively regulate Ngn1/2, its main function at this step is likely the suppression of glutamatergic differentiation pathway. In this model, we hypothesize that Helt expression may oscillate in the cycling midbrain neuroepithelial cells, the cells in the Helt^{high} phase are potent to produce GABAergic neurons in the presence of Ascl1, and Helt expression is rapidly downregulated after the onset of differentiation. Gata2 expression is initiated in the differentiating GABAergic precursors, shortly after the strong Helt and Ascl1 expression wave, and its function is to instruct GABAergic differentiation (Figure 12).

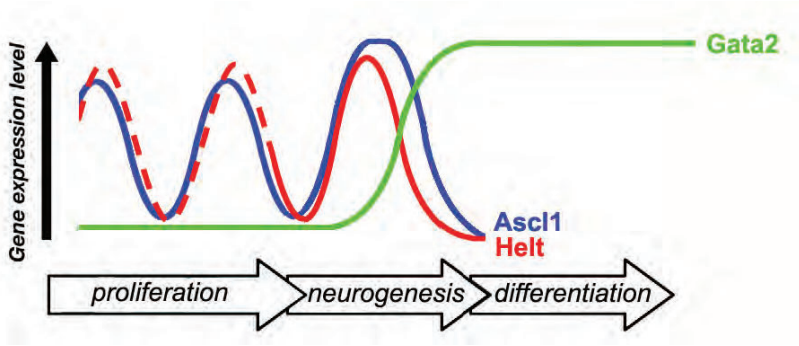

Figure 12. The expression dynamics of Ascl1, Helt and Gata2 during the midbrain GABAergic neuron development. In the proliferationg progenitors, Ascl1 and Helt expression oscillates, probably in response to Hes-mediated inhibition cycles. During neurogenesis, Ascl1 expression stabilizes and Gata2 expression is initiated at the Helt^{high} phase, specifying a GABAergic identity in the newborn neuronal precursor.

5.3. Fate transformation in the Gata2cko midbrain

Gata2 participates in the neuronal subtype selection in several contexts: it is involved in the specification of ventral interneuron precursors in the spinal cord (Peng et al., 2007; Zhou et al., 2000) and serotonergic neurons in the hindbrain (Craven et al., 2004), as well as sympathetic neuron development in peripheral nervous system (Tsarovina et al., 2004). Gata2 is also expressed in more anterior regions of the CNS, like midbrain and diencephalon. We studied the function of Gata2 in these brain regions and showed that it acts as a post-mitotic selector gene for the GABAergic over the glutamatergic neuron fate in the midbrain and dorsal thalamus, but not in the r1.

Using mouse and chick models, we demonstrated that Gata2 is both required and sufficient for the GABAergic neuron development. In the chicken ventral spinal cord,

forced expression of Gata2 can induce Gata3$^+$ V2b fate, simultaneously suppressing Chx10$^+$ V2a fate (Karunaratne et al., 2002), which represents glutamatergic neuron population (Lundfald et al., 2007). Similarly, we showed that Gata2 promotes GABAergic differentiation in the chicken dorsal midbrain cells. Consistently, in the absence of Gata2, the neural precursors in the mouse midbrain appear to undergo a fate transformation and adopt a *Vglut2*$^+$ glutamatergic phenotype.

However, it is possible that the GABA-to-glutamatergic fate switch is still incomplete, as observed in the Helt knockout brains, where dorsal midbrain neurons lose most of the determinants of GABAergic neurotransmission, but maintain GABA transporter-1 expression (Guimera et al., 2006b), simultaneously upregulating glutamatergic neuron markers Pou4f1 and Vglut2 (Nakatani et al., 2007). It may be expected that ectopically produced glutamatergic cells in the Gata2cko are not fully comparable with endogenously produced cells, which have undergone a consummate differentiation pathway while the precursors in prospective GABAergic domains have acquired a distinct competence during the proliferative stages in the VZ. For instance, the Vglut2$^+$ cells in m5 MZ are Nkx6-1$^+$ (alike m6 glutamatergic cells), but do not maintain Lhx1/2, which is also a characteristic of m6. Lhx1/2 as well as Nkx2-2 expression is maintained throughout the m4 in Gata2cko, similar to wt. However, these genes are uniformly expressed in the wt m4, and we can assume their expression involves both Gata2$^+$ GABAergic and Pax6$^+$ glutamatergic precursors. As there are no specific markers for m4-d precursor cells, it is difficult to assess alterations in the subtype-specific patterning in this domain. Regardless to the possible alterations in the HD gene expression, *Vglut2* expression clearly demonstrates the glutamatergic neurotransmitter phenotype acquired by all the *Gata2*-deficient precursor cells in midbrain.

5.4. Multiple functions of Ascl1 in the GABAergic neurogenesis

A complete requirement of Ascl1 for the midbrain GABAergic neuron development had been suggested previously, based on the loss of GABAergic neuron markers Gad2 and GABA (Miyoshi et al., 2004). However, it should be noted that from the data provided in that paper, one can depict Gad2-positive cells in the E11.5 Ascl1KO midbrain, most probably located in m5 or m4 domain (Fig. 8D' in Miyoshi et al., 2004). Our more detailed analysis of the same phenotype revealed that while no GABAergic neurons are produced from dorsal midbrain neuroepithelium in the absence of Ascl1, GABAergic neurogenesis in the ventral midbrain (m3-m5 domains) is markedly delayed, but not abolished. Thus, in the ventral midbrain, Ascl1 seems not to specify the GABAergic neuron fate, but rather to control the timing of their production. Altogether, our results favour the hypothesis of context-dependent functions of Ascl1 in the midbrain.

Also in some other neuronal populations, it has been shown that proneural genes promote the initiation of neurogenesis, without being absolutely required for it. For example, vMB DA neurons require Ngn2 for their normal development. In absence of Ngn2, the neurogenesis in the vMB is delayed, but not completely abolished; resulting in a marked reduction in mature DA neuron numbers (Kele et al., 2006). Similar to our observations in Ascl1KO m3-m5, downregulation of Dll1 and Hes5 accompanies the initial delay in neurogenesis in Ngn2$^{-/-}$ vMB. However, in the vMB, Ascl1 is expressed in

absence of Ngn2 and appears to partially compensate for its function (Kele et al., 2006), a phenomenon we did not observe in the GABAergic neurogenesis in m3-m5. Another example of complex requirements for proneural function can be found in the development of the central (caudal hindbrain) and peripheral noradrenergic neurons. In the hindbrain, the loss of Ascl1 results in a delayed onset of noradrenergic neuron production in the dorsal neural tube and, subsequently, in a marked decrease in the number of neurons eventually produced (Pattyn et al., 2006). Notably, the expression of another proneural gene, Ngn2, in the Ascl1-deficient progenitors could not rescue this delay, indicating that the two proneural factors may possess different activities in these cells.

As opposed to midbrain, the r1 GABAergic neurons do not require Ascl1 function, and develop normally in the Ascl1KO. Recently, in vitro studies have indicated that Ascl1 can promote GABAergic differentiation in neuronal cells derived from midbrain, but not from r1 cells (Jo et al., 2007). This differential competence seems to be encoded in the cofactors interacting with Ascl1 in these cells (Jo et al., 2007).

5.5. Comparison of Gata2, Ascl1 and Helt functions

The GABAergic phenotype observed in Ascl1KO prenatal brain greatly resembles HeltKO described by Guimera et al. (2006b), where the same groups of ventral *Gad1*$^{+}$ cells seem to be unaffected. Also, the unaffected GABAergic neurogenesis is apparently associated with maintained Gata2 expression in both cases. However, there are significant

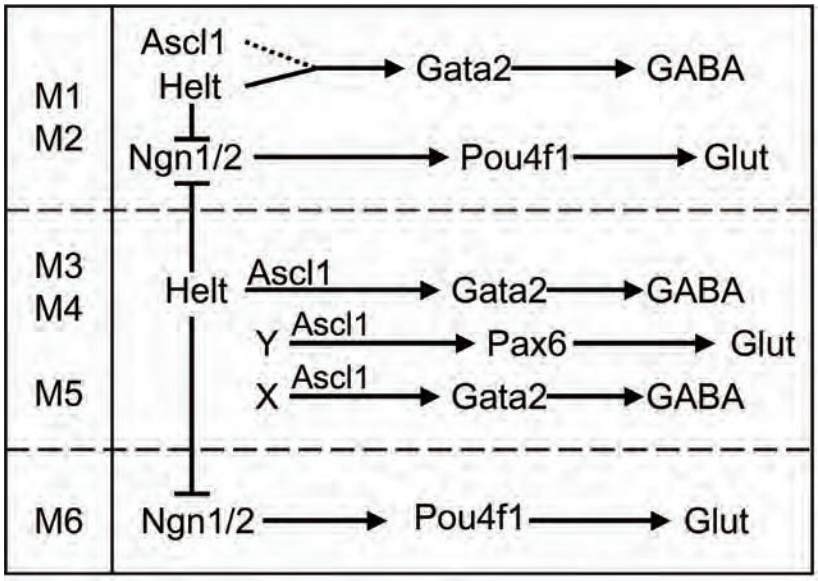

Figure 13. The genetic regulation of GABAergic and glutamatergic neuron development in midbrain neuroepithelium. In the m1-m2 (dorsal midbrain), both Ascl1 and Helt contribute to the specification of GABAergic phenotype by Gata2. In m3-m4, Helt is required for Gata2 expression, while Ascl1 regulates the timing of neurogenesis throughout the m3-m5. The factors that regulate the expression of Gata2 in m5 are unknown. Glutamatergic neurogenesis is controlled by Ngn1/2 and Pou4f1 in m1-m2 and m5. Pax6 is the earliest indicator of glutamatergic neurons in the m4.

differences in the mechanisms affected during midbrain development by the loss Ascl1 or Helt. First, *Ngn2* never expands into the m3-5 domain in the Ascl1KO, as it happens in the HeltKO (Nakatani et al., 2007). Second, the timing of neurogenesis is delayed in the Ascl1KO, but seems to be unaffected in the Helt mutant, where *Gad1* expression can be detected at E11.5, although clearly reduced. Third, in the absence of Helt, prospective GABAergic neurons undergo respecification into a *Vglut2*$^{+}$ glutamatergic fate, which is similar to Gata2cko, but not observed in the Ascl1KO.

In comparison to Helt or Ascl1 mutant mice, Gata2cko phenotype is clearly more severe, lacking dorsal as well as the ventral, mRF and vlPAG GABAergic neurons. The GABAergic neurons associated with the midbrain dopaminergic nuclei VTA and SN are the only subpopulations similarly unaffected by the absence of either Ascl1, Helt or Gata2. Compared to the developmental roles of proneural gene Ascl1 and Ngn1/2 repressor Helt, Gata2 is the factor most directly linked to GABAergic fate acquirement in neuronal precursors. Indeed, in contrast to Ascl1 or Helt, loss of Gata2 does not affect the patterning, proliferation or neurogenic cell cycle exit of GABAergic progenitors, but its function is restricted to the neuronal subtype commitment in the early stage of precursor differentiation. As mentioned before, in absence of either Gata2 or Helt, GABAergic progenitors switch their fate to a glutamatergic pathway, however, the mechanisms of the fate switch appear to be different. In HeltKO, the de-repression of Ngn1/2 allows the specification of ectopic glutamatergic progenitors. In contrast, Gata2-mediated induction of GABAergic differentiation may be achieved by direct transcriptional activation of target genes, while the repression of glutamatergic pathway is likely mediated by currently unidentified downstream effectors.

In summary, while the requirements for Ascl1 and Helt can vary between different cell populations, all midbrain cells seem to exclusively depend on Gata2 in order to commit to GABAergic fate.

5.6. Origin of midbrain GABAergic neurons

The Ascl1KO and Gata2cko analyses provided indirect evidence of the origin of different GABAergic neuron subpopulations in the midbrain. The complete absence of GABAergic neurogenesis in Ascl1KO dorsal midbrain associated with the loss of d/dlPAG and SC/IC GABAergic neurons conspicuously suggests the origin of these cells in m1-m2 neuroepithelium, while the delayed and reduced neurogenesis in ventrolateral midbrain and partial loss of vlPAG and mRF GABAergic neurons indicates the origin of these more ventral nuclei in m3-m5 zones of midbrain. Our results also support the hypothesis that GABAergic neurons in the midbrain reach their final position in adult functional structures predominantly by radial migration, consistent with previous observations (Tan et al., 2002; Tsunekawa et al., 2005).

5.7. Origin and migration of VTA and SNpr GABAergic neurons

The developing brain is genetically and morphologically compartmentalized and it is a general understanding that the neurons located in certain region originate within this

compartment, with few exceptions. For example, the serotonergic neurons in mouse are born in the anterior hindbrain, and migrate anteriorly to populate the midbrain Raphe nuclei later (Scott et al, 2005). Our data indicates that vMB GABAergic neurons might similarly provide an exception of the compartment-restriction view.

Our studies of the genetic regulation of GABAergic neuron development revealed a unique population within the ventral midbrain nuclei: the GABAergic neurons in VTA and SNpr. Even in the Gata2cko midbrains, where all other GABAergic neurons undergo a fate transformation, these cells were developing apparently normally. Gata2 is required for the GABAergic neurogenesis in mb and diencephalon (I, III), but not in r1 (I). Furthermore, the VTA and SNpr GABAergic cells are also present in normal numbers in the midbrains of mice lacking either Ascl1, which is also dispensable for the r1 GABAergic neuron development (II), or Helt, which is expressed in mb, but not in r1 (Guimera et al., 2006a). Therefore, we found it most likely that VTA-SNpr GABAergic neurons originate from the nearest unaffected compartment, r1.

Indeed, by genetic fate mapping, we identified the origin of VTA-SNpr GABAergic neurons in the r1. We have demonstrated the distinct origins of vMB GABAn and all other MB GABAergic neurons, revealed by the comparison of labelling patterns of En1-Cre and Gbx2-CreERT2. All midbrain cells are labelled by En1-Cre, while Gbx2-CreERT2 specifically labels VTA and SNpr GABAergic neurons and not other populations in the midbrain (Fig. 5 F,G in III). The comparison of Shh-Cre and Gbx2-CreERT2 labelling patterns emphasizes the distinct origin of two major classes of neurons in VTA and SN: the r1-derived GABA and midbrain derived DA neurons (Fig. 5 F,G in III). Notably, the SNpc and SNpr can be (anatomically) very differently positioned within the basal ganglia in different species, supporting the notion of a differential origin for these nuclei (Smeets et al., 2000).

We then followed the appearance of the r1-derived vMB GABAn in their final locations. First, we determined the time frame of their production, which showed small, but statistically significant differences compared to other GABAergic neurons in the midbrain. According to our birth-dating, SNpr GABAn are produced before E13.5. However, the first stage where we could conspicuously detect *Gad1*$^{+}$ cells in the VTA-SNpr area, was E15.5. Thus, it seems that the r1-derived vMB GABAn reach their final positions as post-mitotic precursors. Alternatively, these cells might migrate to MB already earlier and initiate the expression of *Gad1* only later, when they mature in their final position. As the appearance of Gbx2-CreERT2-labelled cells in the midbrain correlated with the observed *Gad1* expression, we nevertheless favor the hypothesis of post-mitotic migration.

The cues used for the r1-MB migration of GABAergic neurons are, at present, completely unknown. Because of the close association of DA and GABAergic neurons in the ventral midbrain, and the relevance of dopamine signalling in the migration of forebrain GABAergic neurons (Crandall et al., 2007), it is tempting to speculate that dopamine signalling may regulate the migration of vMB GABAn. However, this hypothesis remains to be studied.

5.8. Protein partners of Gata2 in transcriptional regulation

TAL and FOG proteins associate with GATA TFs and modulate its transcriptional regulator activity (Cantor and Orkin, 2002). Similar to Gata2 (Nardelli et al., 1999), the expression of several Gata2 associated TFs has been recognized in the CNS. For example, Tal1 (Elefanty et al., 1999), Tal2 (Mori et al., 1999) and Fog2 (Lu et al., 1999) are expressed in the mouse midbrain. However, the cell type(s) expressing these TFs in midbrain has not been identified. In the ventral spinal cord, Tal1 appears to be a crucial determinant of inhibitory V2 interneuron fate (Peng 2007).

Our comparisons of Gata2, Tal1, Tal2 and Fog2 expression in the mouse midbrain suggest that all these genes are induced by Gata2 in the midbrain GABAergic neurons, indicating that these TFs are involved at least in some aspects of GABAergic fate specification by Gata2. Consistently, and strikingly enough, the appearance of Tal2 mutant mice greatly resembles Helt (and Gata2) mutant, with no gross anatomical abnormalities in their brains, reduced size and body weight of the young mice and premature death within 6-8 weeks of age (Bucher et al., 2000).

In addition to midbrain, we found that Gata2 and Tal1 are both strongly expressed in the r1. In contrast, Tal2 mRNA levels are near undetectable in the r1 (unpublished results), which may indicate a function in the establishment of differences between MB and r1 GABAergic neuron development. Furthermore, the very similar expression patterns of Gata2 and Tal2 predominantly in the basal side of VZ suggest that these TFs function in a similar stage of neurogenesis. It is probable that rather than a single TF, different transcription factor complexes regulate GABAergic neuron differentiation in different progenitor populations.

5.9. Conclusions and future plans

This work contributes to the understanding of the genetic regulation of GABAergic fate selection in the midbrain neuroepithelium. Also, my results underscore the genetic diversity in the mechanisms regulating GABAergic neuron development in different brain regions, as well as the diversity between the GABAergic neurons within a single compartment, the midbrain.

In the future, studies of Gata2 transcription factor complex and its target genes should further elucidate the mechanisms of GABAergic fate specification by this TF. Our mRNA expression profiling revealed several potential candidates for such studies, including a few potential protein partners of Gata2 in the transcriptional regulation. From these genes, Tal2 is perhaps of special interest as it is expressed in the intermediate zone between VZ and MZ, as well as in scattered VZ cells, which is highly similar to the pattern of Gata2 expression. Based on superficial observations, Tal2 mutant phenotype also greatly resembles Gata2 and Helt mutant, but unfortunately has remained poorly analyzed.

Secondly, we would like to understand the mechanisms of GABAergic neuron development in the ventral r1 and the relocation of GABAergic neurons from the r1 to midbrain. Also, the projection patterns of SNpr and especially VTA GABAergic neurons are currently incompletely described and would deserve greater attention.

5.10. Implications to physiology, behaviour and psychiatric disease

In the brain, wide array of GABAergic neurons are identified, differing in their use of co-neurotransmitters, developmental migratory patterns, final localization and function in the adult structures. Several studies have shown that the diversity of cortical GABAergic interneurons is spatially and genetically encoded in the subpallial neuroepithelium (Cobos et al., 2005; Flames and Marin, 2005; Fogarty et al., 2007). We have provided evidence of diverse genetic mechanisms acting to specify spatially and functionally distinct GABAergic neuron populations in the midbrain and anterior hindbrain.

Besides local inhibitory interneuron function, there are prominent groups of GABAergic projection neurons in the midbrain. These neurons, in synergy with association and limbic areas, are involved in nociception, locomotion, processing of pain and aversive stimuli, learning, reward and addiction. Therefore, understanding the differences between these cell populations provides ground for more specific and effective approaches to the conditions affecting such functions.

SUMMARY

GABAergic neurons are implicated in various behavioural functions and psychiatric disease and are a major neurochemical drug targets. Midbrain GABAergic neurons are involved in the functions as variable as perception of visual stimuli, emotions such as fear and anxiety, and control of body movement. Despite the functional and pharmacological importance of this class of neurons, the understanding of their development is incomplete.

The aim of this work was to investigate the genetic regulation of GABAergic fate selection in the midbrain. We have demonstrated that a zink-finger transcription factor Gata2 is both necessary and sufficient to induce the GABAergic differentiation in the midbrain, but not in anterior hindbrain. We also characterized the complex requirements of proneural transcription factor Ascl1 during midbrain GABAergic neuron development. The results of this study underscore the diversity within midbrain GABAergic neurons.

In addition, we have revealed fundamental differences in the GABAergic neuron production and positioning in the proximity of midbrain dopaminergic nuclei (SN and VTA) in comparison to all other midbrain areas. Specifically, we showed that the SN-VTA region GABAergic neurons originate outside midbrain, in the anterior hindbrain (r1), and suggest that they populate the midbrain only as most-mitotic precursors.

Future studies should further elucidate the mechanisms of genetic and transcriptional regulation of GABAergic neuron development in midbrain and r1.

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REFERENCES

Acampora, D., Avantaggiato, V., Tuorto, F., and Simeone, A. (1997). Genetic control of brain morphogenesis through Otx gene dosage requirement. Development 124, 3639-3650.

Agarwala, S., and Ragsdale, C.W. (2002). A role for midbrain arcs in nucleogenesis. Development 129, 5779-5788.

Agarwala, S., Sanders, T.A., and Ragsdale, C.W. (2001). Sonic hedgehog control of size and shape in midbrain pattern formation. Science 291, 2147-2150.

Akai, J., Halley, P.A., and Storey, K.G. (2005). FGF-dependent Notch signaling maintains the spinal cord stem zone. Genes Dev. 19, 2877-2887.

Altman, J., and Bayer, S.A. (1981). Development of the brain stem in the rat. V. Thymidine-radiographic study of the time of origin of neurons in the midbrain tegmentum. J. Comp. Neurol. 198, 677-716.

Andang, M., Hjerling-Leffler, J., Moliner, A., Lundgren, T.K., Castelo-Branco, G., Nanou, E., Pozas, E., Bryja, V., Halliez, S., Nishimaru, H., *et al.* (2008). Histone H2AX-dependent GABA(A) receptor regulation of stem cell proliferation. Nature 451, 460-464.

Anderson, S.A., Eisenstat, D.D., Shi, L., and Rubenstein, J.L. (1997a). Interneuron migration from basal forebrain to neocortex: dependence on Dlx genes. Science 278, 474-476.

Anderson, S.A., Qiu, M., Bulfone, A., Eisenstat, D.D., Meneses, J., Pedersen, R., and Rubenstein, J.L. (1997b). Mutations of the homeobox genes Dlx-1 and Dlx-2 disrupt the striatal subventricular zone and differentiation of late born striatal neurons. Neuron 19, 27-37.

Andrews, W.D., Barber, M., and Parnavelas, J.G. (2007). Slit-Robo interactions during cortical development. J. Anat. 211, 188-198.

Bayly, R.D., Ngo, M., Aglyamova, G.V., and Agarwala, S. (2007). Regulation of ventral midbrain patterning by Hedgehog signaling. Development 134, 2115-2124.

Beddington, R.S., and Robertson, E.J. (1999). Axis development and early asymmetry in mammals. Cell 96, 195-209.

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.

Behbehani, M.M. (1995). Functional characteristics of the midbrain periaqueductal gray. Prog. Neurobiol. 46, 575-605.

Ben-Ari, Y. (2002). Excitatory actions of gaba during development: the nature of the nurture. Nat. Rev. Neurosci. 3, 728-739.

Ben-Ari, Y., Cherubini, E., Corradetti, R., and Gaiarsa, J.L. (1989). Giant synaptic potentials in immature rat CA3 hippocampal neurones. J. Physiol. 416, 303-325.

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.

Bertrand, N., Castro, D.S., and Guillemot, F. (2002). Proneural genes and the specification of neural cell types. Nat. Rev. Neurosci. 3, 517-530.

Bertrand, N., Medevielle, F., and Pituello, F. (2000). FGF signalling controls the timing of Pax6 activation in the neural tube. Development 127, 4837-4843.

Binns, K.E., and Salt, T.E. (1997). Different roles for GABAA and GABAB receptors in visual processing in the rat superior colliculus. J. Physiol. 504 (Pt 3), 629-639.

Blaess, S., Corrales, J.D., and Joyner, A.L. (2006). Sonic hedgehog regulates Gli activator and repressor functions with spatial and temporal precision in the mid/hindbrain region. Development 133, 1799-1809.

Blaesse, P., Airaksinen, M.S., Rivera, C., and Kaila, K. (2009). Cation-chloride cotransporters and neuronal function. Neuron 61, 820-838.

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.

Boucetta, S., and Jones, B.E. (2009). Activity profiles of cholinergic and intermingled GABAergic and putative glutamatergic neurons in the pontomesencephalic tegmentum of urethane-anesthetized rats. J. Neurosci. 29, 4664-4674.

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.

Brandao, M.L., Anseloni, V.Z., Pandossio, J.E., De Araujo, J.E., and Castilho, V.M. (1999). Neurochemical mechanisms of the defensive behavior in the dorsal midbrain. Neurosci. Biobehav. Rev. 23, 863-875.

Brandao, M.L., Borelli, K.G., Nobre, M.J., Santos, J.M., Albrechet-Souza, L., Oliveira, A.R., and Martinez, R.C. (2005). Gabaergic regulation of the neural organization of fear in the midbrain tectum. Neurosci. Biobehav. Rev. 29, 1299-1311.

Brandao, M.L., Zanoveli, J.M., Ruiz-Martinez, R.C., Oliveira, L.C., and Landeira-Fernandez, J. (2008). Different patterns of freezing behavior organized in the periaqueductal gray of rats: association with different types of anxiety. Behav. Brain Res. 188, 1-13.

Bray, S.J. (2006). Notch signalling: a simple pathway becomes complex. Nat. Rev. Mol. Cell Biol. 7, 678-689.

Briscoe, J. (2009). Making a grade: Sonic Hedgehog signalling and the control of neural cell fate. EMBO J. 28, 457-465.

Briscoe, J., and Novitch, B.G. (2008). Regulatory pathways linking progenitor patterning, cell fates and neurogenesis in the ventral neural tube. Philos. Trans. R. Soc. Lond. B. Biol. Sci. 363, 57-70.

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.

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.

Burch, J.B. (2005). Regulation of GATA gene expression during vertebrate development. Semin. Cell Dev. Biol. 16, 71-81.

Bylund, M., Andersson, E., Novitch, B.G., and Muhr, J. (2003). Vertebrate neurogenesis is counteracted by Sox1-3 activity. Nat. Neurosci. 6, 1162-1168.

Cantor, A.B., and Orkin, S.H. (2005). Coregulation of GATA factors by the Friend of GATA (FOG) family of multitype zinc finger proteins. Semin. Cell Dev. Biol. 16, 117-128.

Cantor, A.B., and Orkin, S.H. (2002). Transcriptional regulation of erythropoiesis: an affair involving multiple partners. Oncogene 21, 3368-3376.

Canty, A.J., Dietze, J., Harvey, M., Enomoto, H., Milbrandt, J., and Ibanez, C.F. (2009). Regionalized loss of parvalbumin interneurons in the cerebral cortex of mice with deficits in GFRalpha1 signaling. J. Neurosci. 29, 10695-10705.

Carr, D.B., and Sesack, S.R. (2000). GABA-containing neurons in the rat ventral tegmental area project to the prefrontal cortex. Synapse 38, 114-123.

Casarosa, S., Fode, C., and Guillemot, F. (1999). Mash1 regulates neurogenesis in the ventral telencephalon. Development 126, 525-534.

Castro, D.S., Skowronska-Krawczyk, D., Armant, O., Donaldson, I.J., Parras, C., Hunt, C., Critchley, J.A., Nguyen, L., Gossler, A., Gottgens, B., Matter, J.M., and Guillemot, F. (2006). Proneural bHLH and Brn proteins coregulate a neurogenic program through cooperative binding to a conserved DNA motif. Dev. Cell. 11, 831-844.

Cau, E., and Blader, P. (2009). Notch activity in the nervous system: to switch or not switch? Neural Dev. 4, 36.

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.

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.

Cheng, L., Samad, O.A., Xu, Y., Mizuguchi, R., Luo, P., Shirasawa, S., Goulding, M., and Ma, Q. (2005). Lbx1 and Tlx3 are opposing switches in determining GABAergic versus glutamatergic transmitter phenotypes. Nat. Neurosci. 8, 1510-1515.

Cheng, Y., Wu, W., Kumar, S.A., Yu, D., Deng, W., Tripic, T., King, D.C., Chen, K.B., Zhang, Y., Drautz, D., *et al.* (2009). Erythroid GATA1 function revealed by genome-wide analysis of transcription factor occupancy, histone modifications, and mRNA expression. Genome Res. 19, 2172-2184.

Cisneros, E., Latasa, M.J., Garcia-Flores, M., and Frade, J.M. (2008). Instability of Notch1 and Delta1 mRNAs and reduced Notch activity in vertebrate neuroepithelial cells undergoing S-phase. Mol. Cell. Neurosci. 37, 820-831.

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.

Colasante, G., Collombat, P., Raimondi, V., Bonanomi, D., Ferrai, C., Maira, M., Yoshikawa, K., Mansouri, A., Valtorta, F., Rubenstein, J.L., and Broccoli, V. (2008). Arx is a direct target of Dlx2 and thereby contributes to the tangential migration of GABAergic interneurons. J. Neurosci. 28, 10674-10686.

Corbin, J.G., Gaiano, N., Machold, R.P., Langston, A., and Fishell, G. (2000). The Gsh2 homeodomain gene controls multiple aspects of telencephalic development. Development 127, 5007-5020.

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.

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.

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., and Richardson, W.D. (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.

Deniau, J.M., Mailly, P., Maurice, N., and Charpier, S. (2007). The pars reticulata of the substantia nigra: a window to basal ganglia output. Prog. Brain Res. 160, 151-172.

Ding, Y.Q., Marklund, U., Yuan, W., Yin, J., Wegman, L., Ericson, J., Deneris, E., Johnson, R.L., and Chen, Z.F. (2003). Lmx1b is essential for the development of serotonergic neurons. Nat. Neurosci. 6, 933-938.

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.

El Wakil, A., Francius, C., Wolff, A., Pleau-Varet, J., and Nardelli, J. (2006). The GATA2 transcription factor negatively regulates the proliferation of neuronal progenitors. Development 133, 2155-2165.

Elefanty, A.G., Begley, C.G., Hartley, L., Papaevangeliou, B., and Robb, L. (1999). SCL expression in the mouse embryo detected with a targeted lacZ reporter gene demonstrates its localization to hematopoietic, vascular, and neural tissues. Blood 94, 3754-3763.

Ericson, J., Muhr, J., Jessell, T.M., and Edlund, T. (1995). Sonic hedgehog: a common signal for ventral patterning along the rostrocaudal axis of the neural tube. Int. J. Dev. Biol. 39, 809-816.

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.

Ferreira, R., Ohneda, K., Yamamoto, M., and Philipsen, S. (2005). GATA1 function, a paradigm for transcription factors in hematopoiesis. Mol. Cell. Biol. 25, 1215-1227.

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.

Flames, N., and Marin, O. (2005). Developmental mechanisms underlying the generation of cortical interneuron diversity. Neuron 46, 377-381.

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.

Fogarty, M., Grist, M., Gelman, D., Marin, O., Pachnis, V., and Kessaris, N. (2007). Spatial genetic patterning of the embryonic neuroepithelium generates GABAergic interneuron diversity in the adult cortex. J. Neurosci. 27, 10935-10946.

Fort, P., Bassetti, C.L., and Luppi, P.H. (2009). Alternating vigilance states: new insights regarding neuronal networks and mechanisms. Eur. J. Neurosci. 29, 1741-1753.

Foster, A.C., and Kemp, J.A. (2006). Glutamate- and GABA-based CNS therapeutics. Curr. Opin. Pharmacol. 6, 7-17.

Gavalas, A., Ruhrberg, C., Livet, J., Henderson, C.E., and Krumlauf, R. (2003). Neuronal defects in the hindbrain of Hoxa1, Hoxb1 and Hoxb2 mutants reflect regulatory interactions among these Hox genes. Development 130, 5663-5679.

George, K.M., Leonard, M.W., Roth, M.E., Lieuw, K.H., Kioussis, D., Grosveld, F., and Engel, J.D. (1994). Embryonic expression and cloning of the murine GATA-3 gene. Development 120, 2673-2686.

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.

Gonzalez-Hernandez, T., and Rodriguez, M. (2000). Compartmental organization and chemical profile of dopaminergic and GABAergic neurons in the substantia nigra of the rat. J. Comp. Neurol. 421, 107-135.

Graham, V., Khudyakov, J., Ellis, P., and Pevny, L. (2003). SOX2 functions to maintain neural progenitor identity. Neuron 39, 749-765.

Groves, A.K., George, K.M., Tissier-Seta, J.P., Engel, J.D., Brunet, J.F., and Anderson, D.J. (1995). Differential regulation of transcription factor gene expression and phenotypic markers in developing sympathetic neurons. Development 121, 887-901.

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., Vogt Weisenhorn, D., Echevarria, D., Martinez, S., and Wurst, W. (2006a). Molecular characterization, structure and developmental expression of Megane bHLH factor. Gene 377, 65-76.

Guimera, J., Weisenhorn, D.V., and Wurst, W. (2006b). Megane/Heslike is required for normal GABAergic differentiation in the mouse superior colliculus. Development 133, 3847-3857.

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.

Hayashi, S., and McMahon, A.P. (2002). Efficient recombination in diverse tissues by a tamoxifen-inducible form of Cre: a tool for temporally regulated gene activation/inactivation in the mouse. Dev. Biol. 244, 305-318.

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.

Heck, N., Kilb, W., Reiprich, P., Kubota, H., Furukawa, T., Fukuda, A., and Luhmann, H.J. (2007). GABA-A receptors regulate neocortical neuronal migration in vitro and in vivo. Cereb. Cortex 17, 138-148.

Heng, J.I., Moonen, G., and Nguyen, L. (2007). Neurotransmitters regulate cell migration in the telencephalon. Eur. J. Neurosci. 26, 537-546.

Henrique, D., Adam, J., Myat, A., Chitnis, A., Lewis, J., and Ish-Horowicz, D. (1995). Expression of a Delta homologue in prospective neurons in the chick. Nature 375, 787-790.

Henrique, D., Hirsinger, E., Adam, J., Le Roux, I., Pourquie, O., Ish-Horowicz, D., and Lewis, J. (1997). Maintenance of neuroepithelial progenitor cells by Delta-Notch signalling in the embryonic chick retina. Curr. Biol. 7, 661-670.

Herberth, B., Minko, K., Csillag, A., Jaffredo, T., and Madarasz, E. (2005). SCL, GATA-2 and Lmo2 expression in neurogenesis. Int. J. Dev. Neurosci. 23, 449-463.

Hernandez-Miranda, L.R., Parnavelas, J.G., and Chiara, F. (2010). Molecules and mechanisms involved in the generation and migration of cortical interneurons. ASN Neuro 2, e00031.

Hidalgo-Sanchez, M., Millet, S., Bloch-Gallego, E., and Alvarado-Mallart, R.M. (2005). Specification of the meso-isthmo-cerebellar region: the Otx2/Gbx2 boundary. Brain Res. Brain Res. Rev. 49, 134-149.

Hirata, H., Yoshiura, S., Ohtsuka, T., Bessho, Y., Harada, T., Yoshikawa, K., and Kageyama, R. (2002). Oscillatory expression of the bHLH factor Hes1 regulated by a negative feedback loop. Science 298, 840-843.

Holmberg, J., Hansson, E., Malewicz, M., Sandberg, M., Perlmann, T., Lendahl, U., and Muhr, J. (2008). SoxB1 transcription factors and Notch signaling use distinct mechanisms to regulate proneural gene function and neural progenitor differentiation. Development 135, 1843-1851.

Horton, S., Meredith, A., Richardson, J.A., and Johnson, J.E. (1999). Correct coordination of neuronal differentiation events in ventral forebrain requires the bHLH factor MASH1. Mol. Cell. Neurosci. 14, 355-369.

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.

Inoue, M., Matsuo, T., and Ogata, N. (1985). Possible involvement of K+-conductance in the action of gamma-aminobutyric acid in the guinea-pig hippocampus. Br. J. Pharmacol. 86, 515-524.

Ishibashi, M., Ang, S.L., Shiota, K., Nakanishi, S., Kageyama, R., and Guillemot, F. (1995). Targeted disruption of mammalian hairy and Enhancer of split homolog-1 (HES-1) leads to up-regulation of neural helix-loop-helix factors, premature neurogenesis, and severe neural tube defects. Genes Dev. 9, 3136-3148.

Ishibashi, M., Leonard, C.S., and Kohlmeier, K.A. (2009). Nicotinic activation of laterodorsal tegmental neurons: implications for addiction to nicotine. Neuropsychopharmacology 34, 2529-2547.

Ito, T., Bishop, D.C., and Oliver, D.L. (2009). Two classes of GABAergic neurons in the inferior colliculus. J. Neurosci. 29, 13860-13869.

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.

Jo, A.Y., Park, C.H., Aizawa, S., and Lee, S.H. (2007). Contrasting and brain region-specific roles of neurogenin2 and mash1 in GABAergic neuron differentiation in vitro. Exp. Cell Res. 313, 4066-4081.

Joksimovic, M., Anderegg, A., Roy, A., Campochiaro, L., Yun, B., Kittappa, R., McKay, R., and Awatramani, R. (2009a). Spatiotemporally separable Shh domains in the midbrain define distinct dopaminergic progenitor pools. Proc. Natl. Acad. Sci. U. S. A. 106, 19185-19190.

Joksimovic, M., Yun, B.A., Kittappa, R., Anderegg, A.M., Chang, W.W., Taketo, M.M., McKay, R.D., and Awatramani, R.B. (2009b). Wnt antagonism of Shh facilitates midbrain floor plate neurogenesis. Nat. Neurosci. 12, 125-131.

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.

Jukkola, T., Lahti, L., Naserke, T., Wurst, W., and Partanen, J. (2006). FGF regulated gene-expression and neuronal differentiation in the developing midbrain-hindbrain region. Dev. Biol. 297, 141-157.

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., and Kobayashi, T. (2007). The Hes gene family: repressors and oscillators that orchestrate embryogenesis. Development 134, 1243-1251.

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.

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.

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.

Kaufling, J., Veinante, P., Pawlowski, S.A., Freund-Mercier, M.J., and Barrot, M. (2010). gamma-Aminobutyric acid cells with cocaine-induced DeltaFosB in the ventral tegmental area innervate mesolimbic neurons. Biol. Psychiatry 67, 88-92.

Kele, J., Simplicio, N., Ferri, A.L., Mira, H., Guillemot, F., Arenas, E., and Ang, S.L. (2006). Neurogenin 2 is required for the development of ventral midbrain dopaminergic neurons. Development 133, 495-505.

Khirug, S., Yamada, J., Afzalov, R., Voipio, J., Khiroug, L., and Kaila, K. (2008). GABAergic depolarization of the axon initial segment in cortical principal neurons is caused by the Na-K-2Cl cotransporter NKCC1. J. Neurosci. 28, 4635-4639.

Kim, E.J., Battiste, J., Nakagawa, Y., and Johnson, J.E. (2008). Ascl1 (Mash1) lineage cells contribute to discrete cell populations in CNS architecture. Mol. Cell. Neurosci. 38, 595-606.

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.

King, A.J. (2004). The superior colliculus. Curr. Biol. 14, R335-8.

Kishi, M., Mizuseki, K., Sasai, N., Yamazaki, H., Shiota, K., Nakanishi, S., and Sasai, Y. (2000). Requirement of Sox2-mediated signaling for differentiation of early Xenopus neuroectoderm. Development 127, 791-800.

Kolk, S.M., Gunput, R.A., Tran, T.S., van den Heuvel, D.M., Prasad, A.A., Hellemons, A.J., Adolfs, Y., Ginty, D.D., Kolodkin, A.L., Burbach, J.P., Smidt, M.P., and Pasterkamp, R.J. (2009). Semaphorin 3F is a bifunctional guidance cue for dopaminergic axons and controls their fasciculation, channeling, rostral growth, and intracortical targeting. J. Neurosci. 29, 12542-12557.

Krueger, K.C., and Deneris, E.S. (2008). Serotonergic transcription of human FEV reveals direct GATA factor interactions and fate of Pet-1-deficient serotonin neuron precursors. J. Neurosci. 28, 12748-12758.

Kunisch, M., Haenlin, M., and Campos-Ortega, J.A. (1994). Lateral inhibition mediated by the Drosophila neurogenic gene delta is enhanced by proneural proteins. Proc. Natl. Acad. Sci. U. S. A. 91, 10139-10143.

Lahlil, R., Lecuyer, E., Herblot, S., and Hoang, T. (2004). SCL assembles a multifactorial complex that determines glycophorin A expression. Mol. Cell. Biol. 24, 1439-1452.

Landry, J.R., Bonadies, N., Kinston, S., Knezevic, K., Wilson, N.K., Oram, S.H., Janes, M., Piltz, S., Hammett, M., Carter, J., *et al.* (2009). Expression of the leukemia oncogene Lmo2 is controlled by an array of tissue-specific elements dispersed over 100 kb and bound by Tal1/Lmo2, Ets, and Gata factors. Blood 113, 5783-5792.

Latasa, M.J., Cisneros, E., and Frade, J.M. (2009). Cell cycle control of Notch signaling and the functional regionalization of the neuroepithelium during vertebrate neurogenesis. Int. J. Dev. Biol. 53, 895-908.

Laviolette, S.R., and van der Kooy, D. (2004a). GABAA receptors signal bidirectional reward transmission from the ventral tegmental area to the tegmental pedunculopontine nucleus as a function of opiate state. Eur. J. Neurosci. 20, 2179-2187.

Laviolette, S.R., and van der Kooy, D. (2004b). The neurobiology of nicotine addiction: bridging the gap from molecules to behaviour. Nat. Rev. Neurosci. 5, 55-65.

Lee, J.E., Hollenberg, S.M., Snider, L., Turner, D.L., Lipnick, N., and Weintraub, H. (1995). Conversion of Xenopus ectoderm into neurons by NeuroD, a basic helix-loop-helix protein. Science 268, 836-844.

Lee, M.S., Rinne, J.O., and Marsden, C.D. (2000). The pedunculopontine nucleus: its role in the genesis of movement disorders. Yonsei Med. J. 41, 167-184.

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.

Letting, D.L., Chen, Y.Y., Rakowski, C., Reedy, S., and Blobel, G.A. (2004). Context-dependent regulation of GATA-1 by friend of GATA-1. Proc. Natl. Acad. Sci. U. S. A. 101, 476-481.

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.

Lillevali, K., Haugas, M., Pituello, F., and Salminen, M. (2007). Comparative analysis of Gata3 and Gata2 expression during chicken inner ear development. Dev. Dyn. 236, 306-313.

Lillevali, K., Matilainen, T., Karis, A., and Salminen, M. (2004). Partially overlapping expression of Gata2 and Gata3 during inner ear development. Dev. Dyn. 231, 775-781.

Lin, L., Rao, Y., and Isacson, O. (2005). Netrin-1 and slit-2 regulate and direct neurite growth of ventral midbrain dopaminergic neurons. Mol. Cell. Neurosci. 28, 547-555.

Lindsell, C.E., Boulter, J., diSibio, G., Gossler, A., and Weinmaster, G. (1996). Expression patterns of Jagged, Delta1, Notch1, Notch2, and Notch3 genes identify ligand-receptor pairs that may function in neural development. Mol. Cell. Neurosci. 8, 14-27.

Liu, A., and Joyner, A.L. (2001). EN and GBX2 play essential roles downstream of FGF8 in patterning the mouse mid/hindbrain region. Development 128, 181-191.

Livesey, F.J., and Hunt, S.P. (1997). Netrin and netrin receptor expression in the embryonic mammalian nervous system suggests roles in retinal, striatal, nigral, and cerebellar development. Mol. Cell. Neurosci. 8, 417-429.

Lopez-Bendito, G., Lujan, R., Shigemoto, R., Ganter, P., Paulsen, O., and Molnar, Z. (2003). Blockade of GABA(B) receptors alters the tangential migration of cortical neurons. Cereb. Cortex 13, 932-942.

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.

Lowry, J.A., and Atchley, W.R. (2000). Molecular evolution of the GATA family of transcription factors: conservation within the DNA-binding domain. J. Mol. Evol. 50, 103-115.

Lu, J.R., McKinsey, T.A., Xu, H., Wang, D.Z., Richardson, J.A., and Olson, E.N. (1999). FOG-2, a heart- and brain-enriched cofactor for GATA transcription factors. Mol. Cell. Biol. 19, 4495-4502.

Lundfald, L., Restrepo, C.E., Butt, S.J., Peng, C.Y., Droho, S., Endo, T., Zeilhofer, H.U., Sharma, K., and Kiehn, O. (2007). Phenotype of V2-derived interneurons and their relationship to the axon guidance molecule EphA4 in the developing mouse spinal cord. Eur. J. Neurosci. 26, 2989-3002.

Machold, R., and Fishell, G. (2005). Math1 is expressed in temporally discrete pools of cerebellar rhombic-lip neural progenitors. Neuron 48, 17-24.

Maricich, S.M., and Herrup, K. (1999). Pax-2 expression defines a subset of GABAergic interneurons and their precursors in the developing murine cerebellum. J. Neurobiol. 41, 281-294.

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

Marin, O., and Rubenstein, J.L. (2001). A long, remarkable journey: tangential migration in the telencephalon. Nat. Rev. Neurosci. 2, 780-790.

Marin, O., Yaron, A., Bagri, A., Tessier-Lavigne, M., and Rubenstein, J.L. (2001). Sorting of striatal and cortical interneurons regulated by semaphorin-neuropilin interactions. Science 293, 872-875.

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.

Matter-Sadzinski, L., Matter, J.M., Ong, M.T., Hernandez, J., and Ballivet, M. (2001). Specification of neurotransmitter receptor identity in developing retina: the chick ATH5 promoter integrates the positive and negative effects of several bHLH proteins. Development 128, 217-231.

McHaffie, J.G., Stanford, T.R., Stein, B.E., Coizet, V., and Redgrave, P. (2005). Subcortical loops through the basal ganglia. Trends Neurosci. 28, 401-407.

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.

Mizuseki, K., Kishi, M., Matsui, M., Nakanishi, S., and Sasai, Y. (1998). Xenopus Zic-related-1 and Sox-2, two factors induced by chordin, have distinct activities in the initiation of neural induction. Development 125, 579-587.

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.

Muroyama, Y., Fujiwara, Y., Orkin, S.H., and Rowitch, D.H. (2005). Specification of astrocytes by bHLH protein SCL in a restricted region of the neural tube. Nature 438, 360-363.

Nakamura, H., and Funahashi, J. (2001). Introduction of DNA into chick embryos by in ovo electroporation. Methods 24, 43-48.

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.

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.

Nordstrom, U., Jessell, T.M., and Edlund, T. (2002). Progressive induction of caudal neural character by graded Wnt signaling. Nat. Neurosci. 5, 525-532.

Nottingham, W.T., Jarratt, A., Burgess, M., Speck, C.L., Cheng, J.F., Prabhakar, S., Rubin, E.M., Li, P.S., Sloane-Stanley, J., Kong-A-San, J., and de Bruijn, M.F. (2007). Runx1-mediated hematopoietic stem-cell emergence is controlled by a Gata/Ets/SCL-regulated enhancer. Blood 110, 4188-4197.

Ogilvy, S., Ferreira, R., Piltz, S.G., Bowen, J.M., Gottgens, B., and Green, A.R. (2007). The SCL +40 enhancer targets the midbrain together with primitive and definitive hematopoiesis and is regulated by SCL and GATA proteins. Mol. Cell. Biol. 27, 7206-7219.

Olander, S., Nordstrom, U., Patthey, C., and Edlund, T. (2006). Convergent Wnt and FGF signaling at the gastrula stage induce the formation of the isthmic organizer. Mech. Dev. 123, 166-176.

Olson, V.G., and Nestler, E.J. (2007). Topographical organization of GABAergic neurons within the ventral tegmental area of the rat. Synapse 61, 87-95.

Ono, Y., Nakatani, T., Sakamoto, Y., Mizuhara, E., Minaki, Y., Kumai, M., Hamaguchi, A., Nishimura, M., Inoue, Y., Hayashi, H., Takahashi, J., and Imai, T. (2007). Differences in neurogenic potential in floor plate cells along an anteroposterior location: midbrain dopaminergic neurons originate from mesencephalic floor plate cells. Development 134, 3213-3225.

Owens, D.F., and Kriegstein, A.R. (2002). Is there more to GABA than synaptic inhibition? Nat. Rev. Neurosci. 3, 715-727.

Pal, S., Cantor, A.B., Johnson, K.D., Moran, T.B., Boyer, M.E., Orkin, S.H., and Bresnick, E.H. (2004). Coregulator-dependent facilitation of chromatin occupancy by GATA-1. Proc. Natl. Acad. Sci. U. S. A. 101, 980-985.

Pandolfi, P.P., Roth, M.E., Karis, A., Leonard, M.W., Dzierzak, E., Grosveld, F.G., Engel, J.D., and Lindenbaum, M.H. (1995). Targeted disruption of the GATA3 gene causes severe abnormalities in the nervous system and in fetal liver haematopoiesis. Nat. Genet. 11, 40-44.

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.

Pascual, M., Abasolo, I., Mingorance-Le Meur, A., Martinez, A., Del Rio, J.A., Wright, C.V., Real, F.X., and Soriano, E. (2007). Cerebellar GABAergic progenitors adopt an external granule cell-like phenotype in the absence of Ptf1a transcription factor expression. Proc. Natl. Acad. Sci. U. S. A. 104, 5193-5198.

Pata, I., Studer, M., van Doorninck, J.H., Briscoe, J., Kuuse, S., Engel, J.D., Grosveld, F., and Karis, A. (1999). The transcription factor GATA3 is a downstream effector of Hoxb1 specification in rhombomere 4. Development 126, 5523-5531.

Patient, R.K., and McGhee, J.D. (2002). The GATA family (vertebrates and invertebrates). Curr. Opin. Genet. Dev. 12, 416-422.

Pattyn, A., Guillemot, F., and Brunet, J.F. (2006). Delays in neuronal differentiation in Mash1/Ascl1 mutants. Dev. Biol. 295, 67-75.

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.

Pattyn, A., Vallstedt, A., Dias, J.M., Samad, O.A., Krumlauf, R., Rijli, F.M., Brunet, J.F., and Ericson, J. (2003). Coordinated temporal and spatial control of motor neuron and serotonergic neuron generation from a common pool of CNS progenitors. Genes Dev. 17, 729-737.

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.

Petilla Interneuron Nomenclature Group, Ascoli, G.A., Alonso-Nanclares, L., Anderson, S.A., Barrionuevo, G., Benavides-Piccione, R., Burkhalter, A., Buzsaki, G., Cauli, B., Defelipe, J., *et al.* (2008). Petilla terminology: nomenclature of features of GABAergic interneurons of the cerebral cortex. Nat. Rev. Neurosci. 9, 557-568.

Poh, A., Karunaratne, A., Kolle, G., Huang, N., Smith, E., Starkey, J., Wen, D., Wilson, I., Yamada, T., and Hargrave, M. (2002). Patterning of the vertebrate ventral spinal cord. Int. J. Dev. Biol. 46, 597-608.

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, L.M., and Jarman, A.P. (2008). Context dependence of proneural bHLH proteins. Curr. Opin. Genet. Dev. 18, 411-417.

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. (2006). Genetic networks controlling the development of midbrain dopaminergic neurons. J. Physiol. 575, 403-410.

Prakash, N., and Wurst, W. (2004). Specification of midbrain territory. Cell Tissue Res. 318, 5-14.

Puelles, E. (2007). Genetic control of basal midbrain development. J. Neurosci. Res. 85, 3530-3534.

Puelles, E., Acampora, D., Lacroix, E., Signore, M., Annino, A., Tuorto, F., Filosa, S., Corte, G., Wurst, W., Ang, S.L., and Simeone, A. (2003). Otx dose-dependent integrated control of antero-posterior and dorso-ventral patterning of midbrain. Nat. Neurosci. 6, 453-460.

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.

Ramain, P., Khechumian, R., Khechumian, K., Arbogast, N., Ackermann, C., and Heitzler, P. (2000). Interactions between chip and the achaete/scute-daughterless heterodimers are required for pannier-driven proneural patterning. Mol. Cell 6, 781-790.

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.

Rivera, C., Voipio, J., and Kaila, K. (2005). Two developmental switches in GABAergic signalling: the K+-Cl- cotransporter KCC2 and carbonic anhydrase CAVII. J. Physiol. 562, 27-36.

Rivera, C., Voipio, J., Payne, J.A., Ruusuvuori, E., Lahtinen, H., Lamsa, K., Pirvola, U., Saarma, M., and Kaila, K. (1999). The K+/Cl- co-transporter KCC2 renders GABA hyperpolarizing during neuronal maturation. Nature 397, 251-255.

Rodriguez, C.I., and Dymecki, S.M. (2000). Origin of the precerebellar system. Neuron 27, 475-486.

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.

Sapin, E., Lapray, D., Berod, A., Goutagny, R., Leger, L., Ravassard, P., Clement, O., Hanriot, L., Fort, P., and Luppi, P.H. (2009). Localization of the brainstem GABAergic neurons controlling paradoxical (REM) sleep. PLoS One 4, e4272.

Saxen, L. (1989). Neural induction. Int. J. Dev. Biol. 33, 21-48.

Schuler, V., Luscher, C., Blanchet, C., Klix, N., Sansig, G., Klebs, K., Schmutz, M., Heid, J., Gentry, C., Urban, L., *et al.* (2001). Epilepsy, hyperalgesia, impaired memory, and loss of pre- and postsynaptic GABA(B) responses in mice lacking GABA(B(1)). Neuron 31, 47-58.

Serafini, R., Valeyev, A.Y., Barker, J.L., and Poulter, M.O. (1995). Depolarizing GABA-activated Cl- channels in embryonic rat spinal and olfactory bulb cells. J. Physiol. 488 (Pt 2), 371-386.

Sgaier, S.K., Lao, Z., Villanueva, M.P., Berenshteyn, F., Stephen, D., Turnbull, R.K., and Joyner, A.L. (2007). Genetic subdivision of the tectum and cerebellum into functionally related regions based on differential sensitivity to engrailed proteins. Development 134, 2325-2335.

Shimojo, H., Ohtsuka, T., and Kageyama, R. (2008). Oscillations in notch signaling regulate maintenance of neural progenitors. Neuron 58, 52-64.

Sillitoe, R.V., Stephen, D., Lao, Z., and Joyner, A.L. (2008). Engrailed homeobox genes determine the organization of Purkinje cell sagittal stripe gene expression in the adult cerebellum. J. Neurosci. 28, 12150-12162.

Simon, H.H., Scholz, C., and O'Leary, D.D. (2005). Engrailed genes control developmental fate of serotonergic and noradrenergic neurons in mid- and hindbrain in a gene dose-dependent manner. Mol. Cell. Neurosci. 28, 96-105.

Sipila, S.T., Huttu, K., Soltesz, I., Voipio, J., and Kaila, K. (2005). Depolarizing GABA acts on intrinsically bursting pyramidal neurons to drive giant depolarizing potentials in the immature hippocampus. J. Neurosci. 25, 5280-5289.

Smeets, W.J., Marin, O., and Gonzalez, A. (2000). Evolution of the basal ganglia: new perspectives through a comparative approach. J. Anat. 196 (Pt 4), 501-517.

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.

Stern, C.D. (2005). Neural induction: old problem, new findings, yet more questions. Development 132, 2007-2021.

Stuhmer, T., Anderson, S.A., Ekker, M., and Rubenstein, J.L. (2002). Ectopic expression of the Dlx genes induces glutamic acid decarboxylase and Dlx expression. Development 129, 245-252.

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.

Tan, K.R., Brown, M., Labouebe, G., Yvon, C., Creton, C., Fritschy, J.M., Rudolph, U., and Luscher, C. (2010). Neural bases for addictive properties of benzodiazepines. Nature 463, 769-774.

Tan, S.S., Valcanis, H., Kalloniatis, M., and Harvey, A. (2002). Cellular dispersion patterns and phenotypes in the developing mouse superior colliculus. Dev. Biol. 241, 117-131.

Tepper, J.M., and Lee, C.R. (2007). GABAergic control of substantia nigra dopaminergic neurons. Prog. Brain Res. 160, 189-208.

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.

Tripic, T., Deng, W., Cheng, Y., Zhang, Y., Vakoc, C.R., Gregory, G.D., Hardison, R.C., and Blobel, G.A. (2009). SCL and associated proteins distinguish active from repressive GATA transcription factor complexes. Blood 113, 2191-2201.

Trokovic, R., Trokovic, N., Hernesniemi, S., Pirvola, U., Vogt Weisenhorn, D.M., Rossant, J., McMahon, A.P., Wurst, W., and Partanen, J. (2003). FGFR1 is independently required in both developing mid- and hindbrain for sustained response to isthmic signals. EMBO J. 22, 1811-1823.

Tsai, F.Y., Keller, G., Kuo, F.C., Weiss, M., Chen, J., Rosenblatt, M., Alt, F.W., and Orkin, S.H. (1994). An early haematopoietic defect in mice lacking the transcription factor GATA-2. Nature 371, 221-226.

Tsarovina, K., Pattyn, A., Stubbusch, J., Muller, F., van der Wees, J., Schneider, C., Brunet, J.F., and Rohrer, H. (2004). Essential role of Gata transcription factors in sympathetic neuron development. Development 131, 4775-4786.

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.

Turner, A.J., and Whittle, S.R. (1983). Biochemical dissection of the gamma-aminobutyrate synapse. Biochem. J. 209, 29-41.

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 Bockstaele, E.J., and Pickel, V.M. (1995). GABA-containing neurons in the ventral tegmental area project to the nucleus accumbens in rat brain. Brain Res. 682, 215-221.

Vernay, B., Koch, M., Vaccarino, F., Briscoe, J., Simeone, A., Kageyama, R., and Ang, S.L. (2005). Otx2 regulates subtype specification and neurogenesis in the midbrain. J. Neurosci. 25, 4856-4867.

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.

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.

Wang, D.D., and Kriegstein, A.R. (2009). Defining the role of GABA in cortical development. J. Physiol. 587, 1873-1879.

Wang, H.L., and Morales, M. (2009). Pedunculopontine and laterodorsal tegmental nuclei contain distinct populations of cholinergic, glutamatergic and GABAergic neurons in the rat. Eur. J. Neurosci. 29, 340-358.

Wang, V.Y., Rose, M.F., and Zoghbi, H.Y. (2005). Math1 expression redefines the rhombic lip derivatives and reveals novel lineages within the brainstem and cerebellum. Neuron 48, 31-43.

Weisheit, G., Gliem, M., Endl, E., Pfeffer, P.L., Busslinger, M., and Schilling, K. (2006). Postnatal development of the murine cerebellar cortex: formation and early dispersal of basket, stellate and Golgi neurons. Eur. J. Neurosci. 24, 466-478.

Wilkinson, D.G., and Green, J. (1990). In situ hybridization and the three-dimensional construction of serial sections. In Postimplantation Mammalian Embryos, Copp, AJ and Cockroft, DL eds., (Oxford, UK: Oxford University Press) pp. 155-155-171.

Wilson, S.I., and Edlund, T. (2001). Neural induction: toward a unifying mechanism. Nat. Neurosci. 4 Suppl, 1161-1168.

Wood, H.B., and Episkopou, V. (1999). Comparative expression of the mouse Sox1, Sox2 and Sox3 genes from pre-gastrulation to early somite stages. Mech. Dev. 86, 197-201.

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.

Wurst, W., and Bally-Cuif, L. (2001). Neural plate patterning: upstream and downstream of the isthmic organizer. Nat. Rev. Neurosci. 2, 99-108.

Yuan, W., Zhou, L., Chen, J.H., Wu, J.Y., Rao, Y., and Ornitz, D.M. (1999). The mouse SLIT family: secreted ligands for ROBO expressed in patterns that suggest a role in morphogenesis and axon guidance. Dev. Biol. 212, 290-306.

Yue, Y., Widmer, D.A., Halladay, A.K., Cerretti, D.P., Wagner, G.C., Dreyer, J.L., and Zhou, R. (1999). Specification of distinct dopaminergic neural pathways: roles of the Eph family receptor EphB1 and ligand ephrin-B2. J. Neurosci. 19, 2090-2101.

Yun, K., Fischman, S., Johnson, J., Hrabe de Angelis, M., Weinmaster, G., and Rubenstein, J.L. (2002). Modulation of the notch signaling by Mash1 and Dlx1/2 regulates sequential specification and differentiation of progenitor cell types in the subcortical telencephalon. Development 129, 5029-5040.

Zervas, M., Blaess, S., and Joyner, A.L. (2005). Classical embryological studies and modern genetic analysis of midbrain and cerebellum development. Curr. Top. Dev. Biol. 69, 101-138.

Zhong, W., and Chia, W. (2008). Neurogenesis and asymmetric cell division. Curr. Opin. Neurobiol. 18, 4-11.

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.

Zordan, P., Croci, L., Hawkes, R., and Consalez, G.G. (2008). Comparative analysis of proneural gene expression in the embryonic cerebellum. Dev. Dyn. 237, 1726-1735.