GATA transcription factors and their co-regulators guide the development of GABAergic and serotonergic neurons in the anterior brainstem

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List of original publications

The current thesis is based on two original articles and two manuscripts which will be referred to as Roman numerals (I-IV) in the text. The articles I and II are printed by permission from Development.

I. Lahti L., Haugas M., **Tikker L.**, Airavaara M., Voutilainen M.H., Anttila J., Kumar S., Inkinen C., Salminen M., Partanen J. (2016) Differentiation and molecular heterogeneity of inhibitory and excitatory neurons associated with midbrain dopaminergic nuclei. *Development*. Feb 1;143(3):516-29.

II. Haugas M.*, **Tikker L.***, Achim K., Salminen M., Partanen J. (2016) Gata2 and Gata3 regulate the differentiation of serotonergic and glutamatergic neuron subtypes of the dorsal raphe. *Development*. Dec 1;143(23):4495-4508.

III. Morello F.*, Borshagovski D.*, Survila M.*, **Tikker L.***, Kirjavainen A., Estartús N., Knaapi L., Lahti L., Mazutis L., Delogu A., Salminen S., Partanen J. Molecular fingerprint and developmental regulation of the tegmental GABAergic and glutamatergic neurons derived from the anterior hindbrain. *Submitted*.

IV. **Tikker L.**, Casarotto P., Biojone C., Piepponen P., Estartús N., Seelbach A., Laukkanen L., Castrén E., Partanen J. Inactivation of the GATA cofactor ZFPM1 results in abnormal development of dorsal raphe serotonergic neuron subtypes and increased anxiety-like behaviour. *Submitted*.

*these authors contributed equally to the work

Contributions:

Study I: L.T. performed the chicken *in ovo* electroporation experiments, participated in the mouse gene expression studies, analysed the data and made a major contribution to the preparation of figures.

Study II: L.T. participated in the experimental design and analysis of the serotonergic neuron subtype markers. L.T. conducted the experiments with the Cre-reporter mouse lines, neuronal birth-dating, and *Gata3*^{CKO} as well as *Gata2*^{CKO};*Gata3*^{CKO} mouse mutants. L.T. made a major contribution to the analysis of the data, preparation of the figures, and writing of the manuscript.

Study III: L.T. carried out the experiments involving VTg and DTg development, *Zfpm2*^{CKO} and *Sox14*^{GFP} mouse mutants and birth-dating. L.T. made a major contribution to the validation of the scRNAseq results, analysis of the data, preparation of figures and writing of the manuscript.

Study IV: L.T. participated in the experimental design and conducted most of the experiments including *Zfpm1* expression studies and analysis of *Zfpm1*^{CKO} and *Zfpm1*^{CKO};*Zfpm2*^{CKO} mutant mice. L.T. was involved in the studies of *Zfpm1*^{CKO} mouse behaviour. L.T. analysed the data, prepared all the figures and wrote the first draft of the manuscript.

Abbreviations

5-HT	5-hydroxytryptamine, serotonin
bHLH	basic helix-loop-helix protein family
CLP	common lymphoid progenitor
CMP	common myeloid progenitor
CNS	central nervous system
DR	dorsal raphe
DRD	dorsal part of dorsal raphe
DRVL	ventrolateral part of dorsal raphe
DTg	dorsal tegmental nucleus
E	embryonic day
GMP	granulocyte/macrophage progenitor
HSC	hematopoietic stem cell
IPN	interpeduncular nucleus
KO	knock-out
LDTg	laterodorsal tegmental nucleus
MEP	megakaryocyte/erythrocyte progenitor
MHP	median hinge point
NPB	neural plate border
P1	pretectum
P2	thalamus
P3	prethalamus
r	rhombomere
RMTg	rostromedial tegmental nucleus
rp2	rhombencephalic progenitor domain 2
rp3	rhombencephalic progenitor domain 3
rpvMN	rhombencephalic visceral motor neuron progenitor domain
rV2	rhombencephalic V2 domain
rV3	rhombencephalic V3 domain
rvMN	rhombencephalic vMN domain
scRNAseq	single-cell RNA sequencing
SNpc	substantia nigra pars compacta
SNpr	substantia nigra pars reticulata
TF	transcription factor
VTA	ventral tegmental area
VTg	ventral tegmental nucleus

ABSTRACT

The anterior hindbrain segment rhombomere 1 (r1) generates in its ventral (basal) region GABAergic and serotonergic neurons that give rise to various nuclei in the adult brainstem that participate in the modulation of mood and motivation. This thesis focuses on the function of specific transcription factors (TFs) and their co-regulators that control the differentiation of r1-derived neurons and their subtypes.

First, we characterized early ventral r1 development to determine which neurons are generated in this area. In general, the developing neural tube can be divided dorso-ventrally into multiple progenitor domains that have distinct gene expression patterns and give rise to diverse cell types. We found that ventral r1 contains at least three different progenitor domains. Using genetic fate mapping in the mouse, we determined that the two most ventral progenitor domains (Nkx2-2^{+} rp3 and rpvMN) produce serotonergic neurons and oligodendrocytes, whereas the more dorsal progenitor domain (Nkx6-1^{+} rp2) generates GABAergic and glutamatergic neurons. By combining single-cell RNA sequencing (scRNAseq) and expression analyses of subtype-specific TFs, we show that embryonic ventral r1 contains molecularly distinct populations of post-mitotic GABAergic and glutamatergic precursors. We further report that GABAergic neurons from ventral r1 give rise to multiple GABAergic nuclei in the anterior brainstem, such as the posterior substantia nigra pars reticulata (pSNpr), rostromedial tegmental nucleus (RMTg) and ventral tegmental nucleus (VTg), whereas GABAergic neurons of the dorsal tegmental nucleus (DTg) originate from progenitors located in dorsal region of r1.

Second, we analysed the functions of GATA TFs and their regulators in the development of r1-derived GABAergic neurons by conditional mouse mutagenesis. We showed that GATA2 and GATA3, together with their co-factor TAL1, act as neuron-type selectors in early post-mitotic precursors to promote a GABAergic over glutamatergic neuron fate. Analysis of these mutants during later developmental stages showed that GABAergic neurons in the pSNpr, RMTg and VTg were absent, while the number of glutamatergic neurons was increased in other nuclei such as the interpeduncular nucleus (IPN) and the laterodorsal tegmental nucleus (LDTg). We found that ZFPM1 and ZFPM2, two GATA cofactors, are also expressed in GABAergic neuron precursors in r1. However, ZFPM2 does not function as a neuron-type selector in these cells, but rather is required for the proper development of pSNpr, RMTg and VTg GABAergic neurons.

Finally, we studied the role of GATA2, GATA3 and ZFPM1 in the development of dorsal raphe (DR) serotonergic neurons in r1. Conducting overexpression experiments in chicken embryos, we demonstrated that GATA2 and GATA3 guide the differentiation of serotonergic neurons in the absence of their TAL1 partner, which is vital for GABAergic differentiation. We determined that GATA2 acts as a neuron-type selector and is important for all post-mitotic serotonergic neuron precursors to acquire serotonergic identity, whereas GATA3 is required for the differentiation of specific subtype of serotonergic neurons from r1. In addition, GATA2 and GATA3 are necessary for the development of

non-serotonergic glutamatergic neurons in the DR. It was further shown that the GATA cofactor ZFPM1 is essential for the correct development of serotonergic neurons in DR subregions DRVL (ventrolateral part of dorsal raphe) and DRD (dorsal part of dorsal raphe). Loss of ZFPM1 function resulted in increased anxiety-like behaviour and elevated contextual fear memory, a phenotype that was alleviated by chronic treatment with fluoxetine, a selective serotonin reuptake inhibitor (SSRI).

In conclusion, this work reveals neuronal subtypes present in and mechanisms involved with anterior brainstem development and that are important in the determination of behavioural phenotypes. Furthermore, it demonstrates that a complex gene regulatory system, where functions of GATA family selector TFs are modulated by their cofactors, is employed to achieve cell diversity in the central nervous system, mechanisms of which share marked similarities with cell fate determination programs in other developing tissues, such as the hematopoietic system.

1. INTRODUCTION

The adult brain is a complex structure composed of abundantly diverse cell types such as neurons and glial cells [1]. This heterogeneity is achieved during embryonic development when distinct cell types are generated both spatially and temporally throughout the neural tube. The neural tube is a transient structure that contains progenitor cells that give rise to almost all cells of the central nervous system (CNS) [2]. During development, it is patterned on its anterior-posterior and dorsal-ventral axes into multiple, distinct areas that are able to generate specific neuronal types in precise temporal order, which then give rise to discrete brain structures [3]. The neuronal fates each progenitor domain is capable of determining is specified by defined molecular events. When these occur, gene expression is altered by external signals that trigger each region to initiate the production of a unique combination of transcription factors (TFs) required for the development of certain cell types [4].

Following exit from the cell-cycle, these TFs are responsible for the activation of additional TFs needed for differentiation into mature neurons. In the development of some neuronal types, it has been shown that the fate of immature post-mitotic precursors is determined by specific selector genes [5-7]. These encode for TFs that are expressed soon after exit from the cell-cycle and guide neuron development towards a unique identity while often simultaneously repressing alternative fates [8-10].

During the differentiation process, neurons start to express a combination of genes characteristic to certain cell types e.g. genes required to synthesise, transport and metabolise specific neurotransmitters. Furthermore, after neurons have matured, their unique neuronal identity needs to be actively maintained by terminal selector genes [11]. These positively regulate the expression of genes specific to that neuron type, such that when their expression is lost, they are unable to sustain their identity whereas the expression of pan-neuronal genes is not affected [7].

Understanding the expression and function of selector genes and their cofactors is instrumental in understanding CNS development and the generation of neuronal diversity.

2. LITERATURE REVIEW

2.1. GATA TFs and their co-regulators

2.1.1. GATA TFs

In vertebrates, the GATA-family contains six TFs (GATA1-6), all of which recognize the DNA motif WGATAR (W=A/T; R=A/G) [12]. The first GATA factor to be discovered was GATA1 in the late 1980s, when it was shown that erythrocytes contain a protein that binds the regulatory elements of globin genes (e.g., *HBB, Hemoglobin subunit beta*) in the chicken [13]. GATA factors are between 404-589 amino acid long proteins (in mouse) that contain two conserved C2C2-type zinc-finger domains that are termed N-finger (towards N-terminus) and C-finger (towards C-terminus) based on their position (Figure 1) [14-19]. Although both domains are able to bind DNA, the C-finger binds the consensus sequence WGATAR with high affinity, while the N-finger binds DNA weakly and has greater preference for the non-consensus sequence GATC. Additionally, the N-finger functions to stabilize DNA binding and facilitates binding of GATA TFs to their co-regulators [20]. The consensus sequence WGATAR is abundantly present in the genome (approximately one per 1024 base pairs of DNA) yet only some are occupied by the GATA factors [21, 22].

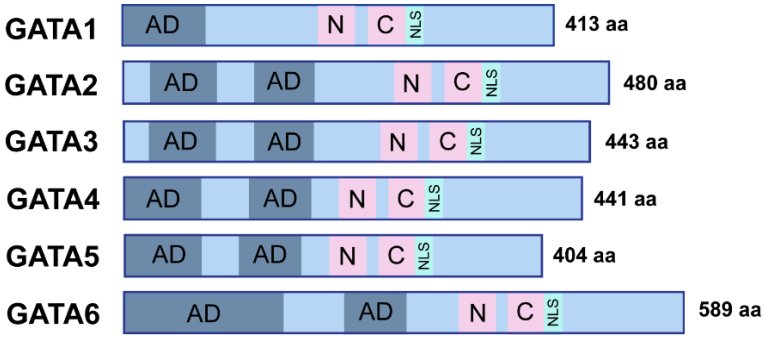

Figure 1. GATA TFs in mouse contain two zinc-finger domains (N and C), nuclear localization signal (NLS) and activation domains (AD). Modified from [23].

The main function of GATA TFs is to regulate the transcription of target genes. They are able to activate or repress transcription and therefore control various developmental processes. In addition, several accessory proteins (discussed below) directly bind or assemble with GATA TFs into protein complexes. These regulate transcription by influencing GATA TFs binding to DNA, affecting chromatin looping, or by summoning additional proteins responsible for chromatin remodelling. GATA factors are divided into two groups based on their expression pattern and their involvement in the development of different tissues. GATA1-3 are expressed in hematopoietic precursors and are required for the differentiation of blood cells. GATA4-6 control the development of various other tissues and organs. For example, GATA4 is necessary for the formation of the heart tube and atrioventricular valves, sex

determination, testis development, and proliferation of epithelium cells in the intestine [24-27]. Inactivation of GATA TFs (except GATA5) causes early embryonic lethality and knock-out (KO) animals die between E6.5-E12 (embryonic day) due to defective haematopoiesis, internal bleeding or abnormal development of the neural tube, heart or endoderm [16, 28-32].

2.1.1.1. Functions and mechanisms of GATAs in cell differentiation: haematopoiesis as a paradigm

The function of GATA TFs and how they are involved in different developmental processes is best characterised in haematopoiesis. During haematopoiesis, multipotent progenitors called haematopoietic stem cells (HSCs) generate multiple different cell types of the blood and immune system. How HSCs are able to give rise to several progenitors that differentiate into numerous distinct cell types has been under intense investigation and GATA TFs GATA1, GATA2, GATA3 as well as some of their co-regulators have been shown to play a significant role, however, their involvement in neuronal differentiation has received less attention. Understanding the function of GATA TFs and their regulators in haematopoiesis may lead to key insights into how these proteins regulate cell fate and differentiation in other tissues.

The first definitive HSCs develop from epithelial cells in the dorsal aorta of embryonic aorta-gonad-mesonephros (AGM) [33]. These cells go through endothelial-to-haematopoietic transition (EHT) and migrate first to the fetal liver and then subsequently reside in adult bone marrow [34]. At E9.5, *Gata2* is expressed in the AGM and is necessary for the initial generation of HSCs where it induces EHT [35-37]. During later stages of development, *Gata2* is responsible for the survival of HSCs [37].

HSCs give rise to multiple different oligopotent progenitors that differentiate into distinct cell types in the blood (Figure 2) [38], such as common lymphoid progenitors (CLP) and common myeloid progenitors (CMP). CMP cells in turn generate megakaryocyte/erythrocyte progenitors (MEP) and granulocyte/macrophage progenitors (GMP). CLPs differentiate into T- and B-lymphocytes; MEPs into megakaryocytes (platelets) and erythrocytes; and GMPs into macrophages and granulocytes (basophils, eosinophils, neutrophils and mast cells). GATA1-3 are necessary for the differentiation of some of these cell types (Figure 2). *Gata2* expression is maintained in CMP and MEP progenitors and is needed to generate MEP progenitors. It also participates in the first stages of erythrocyte differentiation where it induces the expression of genes required for their specification, including *Gata1* [39].

Gata1 is expressed in HSCs and helps determine CMP fate over CLP [40] but is also important for the differentiation and maturation of erythrocytes, megakaryocytes, eosinophils, basophils and mast cells [41, 42]. GATA1 promotes the differentiation and maturation of erythrocytes by down-regulating *Gata2* expression in MEP progenitors and in subsequent immature erythrocyte precursors, where its expression is normally positively self-regulated. GATA1 displaces GATA2 from its auto-regulatory region ("GATA switch"), after which the histone acetyltransferase CREB-binding protein (CBP) is no longer recruited to the region and is dissociated from the site. Consequently, histones are deacetylated

and chromatin remodelling induced, eventually resulting in the repression of *Gata2* expression [22]. In *Gata1* KO mice, erythrocytes are arrested early in the proerythroblast stage and are unable to mature into fully developed erythrocytes [28]. Similarly, progenitors of megakaryocytes over proliferate in *Gata1*-deficient animals and only a few are capable of differentiating into mature platelets [43]. Furthermore, ectopic expression of *Gata1* in GMP and CLP progenitors can redirect them into a megakaryocyte/erythrocyte lineage fate [44].

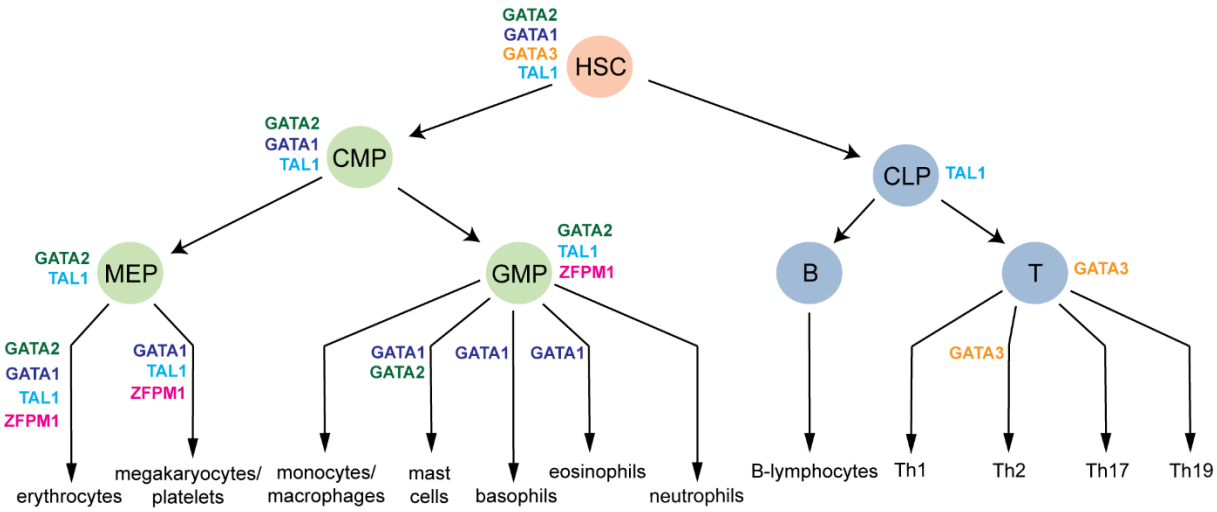

Figure 2. GATA TFs and their co-regulators in haematopoiesis**.** HSC, haematopoietic stem cell; CMP, common myeloid progenitor; CLP, common lymphoid progenitor; MEP, megakaryocyte-erythrocyte progenitors; GMP, granulocyte-macrophage progenitors; B, B-lymphocyte precursor; T, T-lymphocyte precursor.

Like *Gata2*, *Gata3* is also expressed in HSCs and is necessary to maintain normal levels as it has been shown that fewer enter the cell-cycle in *Gata3* KO embryos [45, 46]. CLPs generate B- and T-lymphocytes. B-lymphocytes differentiate in the bone morrow but fully mature in the spleen and lymph nodes. However, precursors of T-lymphocytes migrate to the thymus where they become thymocytes, and subsequently differentiate and mature. During differentiation, thymocytes go through several stages: the four DN (double negative) stages (DN1-4, $CD4^-$,$CD8^-$), DP (double positive) stage ($CD4^+$,$CD8^+$) and then acquire separate $CD4^+$ or $CD8^+$ fates. $CD4^+$ cells then differentiate further into multiple T-helper cells (Th1, Th2, Th17 and Th19). The choice between acquiring a B- or T-lymphocyte fate is determined by GATA3. In progenitors where *Gata3* expression is repressed by the EBF1 (Early B-cell factor 1), a B-lymphocyte lineage is committed to [47]. However, *Gata3* expression is required for the specification of T-lymphocytes and their differentiation to the multiple T-helper cells. In *Gata3* KO mice, T-lymphocyte precursors become arrested in the DN2 stage and maintain the capability to differentiate into B-cells, while normally the DN2 stage thymocytes lose this ability [48]. Furthermore,

GATA3 is necessary for fate determination of Th2 over Th1, and for the maintenance of Th2 identity [49]. *Gata3* is expressed in $CD4^+$ as well as Th2 cells and determines correct Th2-specific cytokine (*IL-4*, *IL-5*, *IL-10*) expression. In addition, Th1 lineage and its specific gene expression (e.g. *Ifng*, *Interferon gamma*) is also determined by TBX21 (T-bet) [50, 51]. Simultaneously, they both repress genes required for the alternative fate. Interestingly, *Gata3* is also expressed in Th1 cells but in these cells, GATA3 is absent from the distal regulatory sites of Th2-specific genes and is instead redistributed to the regulatory sites of Th1-specific genes, where binding of GATA3 is controlled by TBX21 [52].

2.1.2. TAL1 and TAL2

GATA TFs interact either directly or indirectly with several proteins that modulate their specificity and binding. Two of these are TAL1 and TAL2 (T-cell acute lymphocytic leukaemia 1 and 2) which were first discovered in T-cell acute lymphoblastic leukaemia (T-ALL) patients where the disease was determined to be caused by their overexpression as a result of chromosomal translocation [53-55]. TAL1 and TAL2 are 329 and 108 amino acid long proteins (in mouse) that belong to basic helix-loop-helix (bHLH) family of TFs. TAL1 and TAL2 are unable to bind DNA as a homodimers and need to form heterodimers with other bHLH TFs in order to associate with DNA on the E-box sequence (CANNTG) [56, 57].

Like GATA TFs, TAL1 is an important regulator of haematopoiesis and *Tal1*-deficient embryos die between E8.5-E10.5 [58]. It is required for the generation of all blood cell lineages as in chimeric animals, $Tal1^{-/-}$ cells do not contribute to any hematopoietic cells, but are able to give rise to other tissues. This suggests that TAL1 is required for HSC development or the subsequent differentiation of blood cells [59]. However, its role in HSCs remains controversial [60]. In adults, *Tal1* is expressed in HSCs, CMP and MEP progenitors, erythrocyte precursors and mature erythrocytes. It is also expressed in CLP and GMP progenitors but is soon down-regulated and absent from cell types derived from them. Yet, it has been shown to be ectopically expressed in T-cell precursors in T-ALL disease [61]. TAL1 is required for the differentiation of erythrocytes and megakaryocytes [62, 63]. Interestingly, occupancy of TAL1 on DNA changes substantially during the differentiation of these cell lineages as well as between the cell types. In progenitor cells, TAL1 target genes are associated with haematopoiesis (*Kit*, *KIT proto-oncogene receptor tyrosine kinase* and *Gata2*) and proliferation (*VegfA*, *Vascular endothelial growth factor A*), while in the erythrocyte and megakaryocyte precursors, TAL1 target genes are erythrocyte- (*Fech*, *Ferrochelatase*) or megakaryocyte-specific (*Pf4*, *Platelet factor 4*). In addition, TAL1 occupancy is highly influenced by GATA1 and GATA2 [64, 65].

TAL1 and GATA TFs can interact on DNA sites where the E-box sequence is 9 base pairs upstream from WGATAR motifs. There, they form a protein complex that includes other members. For example, the heterodimer of TAL1 and TCF3 (Transcription factor E2-alpha, also known as E2A) located on the E-box binds the proteins LMO2 (LIM domain only 2) and LDB1 (LIM domain-binding

protein 1). LDB1 in turn binds LMO2 and facilitates chromatin looping of the locus control region (LCR) enhancer to the *β-globin* locus. With this configuration established, gene activation is initiated [66]. Furthermore, LMO2 can also associate with GATA1 located on the WGATAR site and connect TAL1 and GATA1 into a single regulatory complex [67]. This protein aggregate (TAL1/TCF3/LMO2/LDB1/GATA1) can control the transcription of erythrocyte-specific genes during erythropoiesis (Figure 4) [68]. Interestingly, in erythrocyte progenitors, there are approximately 15 000 TAL1 occupied DNA sequences, 10 000 WGATAR motifs bound by GATA, and 3500 sites co-occupied by both TAL1 and GATA1 [69]. This indicates that GATA1 and TAL1 can operate independently as well as cooperatively to specify gene expression programs required for erythropoiesis.

Unlike *Tal1*, *Tal2* is not normally expressed in nor is necessary for the development of blood cells. Rather, it is expressed in the embryonic neural tube and is needed for its development. *Tal2* KO animals demonstrate severe developmental defects in the dorsal midbrain and die before adulthood due to an increased accumulation of cerebrospinal fluid (CSF) in the brain [10, 70].

2.1.3. ZFPM1 and ZFPM2

GATA TFs can also be bound by cofactors i.e. transcriptional regulators that do not bind the DNA but are still able to affect transcription. In mammals, two GATA cofactors have been identified: ZFPM1 and ZFPM2 (Zinc finger protein, FOG (Friend of GATA) family member 1 and 2). ZFPM1 and ZFPM2 are 995 and 1151 amino acid long zinc-finger proteins in mouse. ZFPM1 has five C2HC-type and four C2H2-type zinc-finger domains, whereas ZFPM2 contains five C2HC-type and three C2H2-type zinc-finger domains (Figure 3) [71, 72]. ZFPM1 and ZFPM2 do not bind DNA directly but are capable of interacting with GATA TF N-fingers through their C2HC-type finger domains (except number 7) [73]. In many developing tissues, there is a clear overlap in their expression. Both *Zfpm1* and *Zfpm2* are expressed in the embryonic heart and testis, where they cooperate with GATA TFs to influence gene expression and differentiation [72, 74, 75]. However, only *Zfpm1* is required for haematopoiesis and KO embryos die between E10.5-E12.5 due to severe anaemia [76]. Similarly, *Zfpm2*-deficent embryos exhibit abnormal heart development and die around E12.5-E15.5 [77].

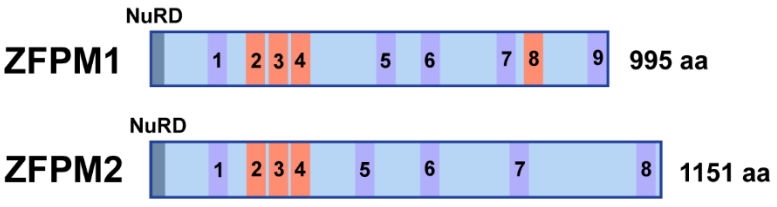

Figure 3. Zinc-finger proteins ZFPM1 and ZFPM2 in mouse. Both contain C2HC-type (purple) and C2H2-type (orange) zinc-finger domains. The interaction site with NuRD complex is shown (NuRD). Modified from [78].

Zfpm1 is expressed in erythrocyte and megakaryocyte precursors, where interaction between ZFPM1 and GATA1 is required for their differentiation [71]. ZFPM1 can interact with GATA TFs and facilitate both the activation and repression of target genes. In erythrocyte precursors, ZFPM1 binding to GATA1 regulates the chromatin occupancy of GATA1 on the *Gata2* locus and is necessary for the "GATA switch" and repression of *Gata2* [79]. However, when GATA2 occupies *Gata2* locus and activates its own transcription, ZFPM1 is not needed. This explains how ZFPM1 regulates chromatin occupancy of GATA TFs on some sites but not others. This was further proven by analysing the chromatin occupancy of GATA1 in *Gata1*-deficient MEP cells that were transfected with either wild-type *Gata1* or *GATA1*^{V205G} (GATA1 that is unable to interact with ZFPM1) sequences. GATA1 occupancy on the DNA changes markedly when GATA1 protein is incapable to associate with ZFPM1 (GATA1^{V205G}). In the absence of GATA1 and ZFPM1 interaction, GATA1 can bind to sites where the binding is usually repressed by ZFPM1. Furthermore, GATA1 is missing from those sites where its association is normally promoted by ZFPM1 [80]. As a result, genes required for the megakaryocyte and erythrocyte differentiation are down-regulated, demonstrating that ZFPM1 facilitated occupancy of GATA1 influences gene transcription.

ZFPM1 interaction with GATA TFs is only required in some cell types. For example, *Zfpm1* is also expressed in GMPs but is soon down-regulated and undetectable in mast cell progenitors. However, *Gata1* and *Gata2* are expressed in mast cell progenitors and are required for their differentiation, indicating that they regulate gene expression in mast cells independently of ZFPM1. Furthermore, ectopic over-expression of *Zfpm1* in these cells divert the cell identity of mast cell precursors towards an erythrocyte lineage [81]. This suggests that GATA TFs are able to regulate transcription ZFPM1-dependently and independently depending on target gene and cell type.

In addition to its function in regulating the chromatin occupancy of GATA TFs, ZFPM1 can also interact and summon a NuRD (Nucleosome remodelling and deacetylase) complex that contains several different subunits that facilitate chromatin remodelling (including histone deacetylation) to regulate transcription [82]. The NuRD complex binds the GATA1/ZFPM1 complex through an N-terminal NuRD motif on ZFPM1 and plays an important role in the repression or activation of some GATA1 target genes needed for the erythropoiesis, such as *Kit* and *Gata2* (repression) or *Hbb-b1* (*Hemoglobin, beta adult major chain*) and *Hba-a1* (*Hemoglobin alpha, adult chain 1*) (activation) [83, 84]. In addition to erythropoiesis, interaction between ZFPM1 and NuRD is also essential for the commitment of MEPs to erythrocyte/megakaryocyte lineages, repression of mast cell-specific genes, and differentiation of megakaryocytes [85]. Since GATA1 is able to simultaneously bind ZFPM1 and LMO2 through its N-finger, ZFPM1 can also incorporate into the regulatory TAL1/TCF3/LMO2/LDB1/GATA1 protein complex and also possibly recruit NuRD (Figure 4) [86].

In conclusion, like GATA TFs, GATA co-regulator TAL1 and cofactor ZFPM1 are essential in haematopoiesis. Both are indispensable for the differentiation of erythrocytes and megakaryocytes. In these cells, ZFPM1 can interact directly with GATA TFs, while TAL1 associates with DNA and connects to GATA through auxiliary proteins. Interaction of GATA with both proteins (not necessarily at the same time) is needed at the regulatory regions of some genes to achieve correct gene expression, while at other sites GATA TFs can act independently of TAL1 and ZFPM1. On sites where the presence of ZFPM1 is required, ZFPM1 is responsible for regulating the chromatin occupancy of GATA and recruiting additional proteins required for the chromatin remodelling.

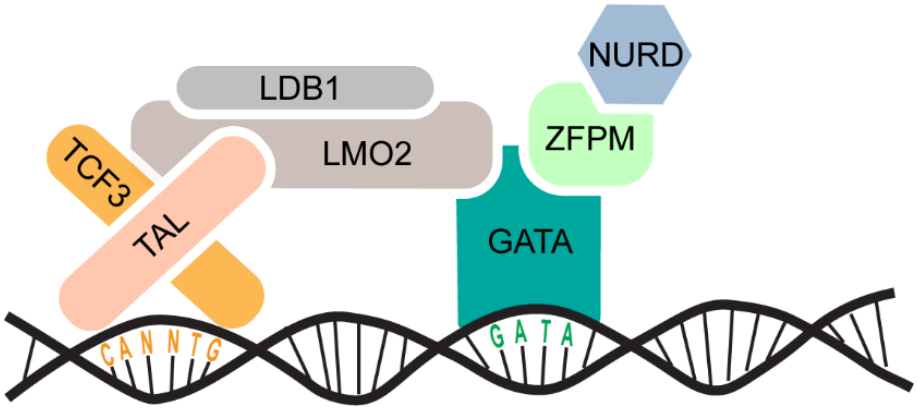

Figure 4. GATA TFs interact with their co-regulators on some WGATAR DNA sequences to form a protein complex that controls transcription.

2.2. Determination of neuronal cell fate and diversity in the central nervous system

The mature CNS is highly heterogeneous and consists of neurons and supporting glial cells such as astrocytes, microglia and oligodendrocytes. The adult mouse brain contains approximately 67 million neurons (human: ~86 billion) that can be subcategorized into distinct neuronal cell types, such as GABAergic, serotonergic, glutamatergic, cholinergic and dopaminergic neurons based on the neurotransmitter they use for signalling [1]. In addition, all of these neuronal cell types can be further divided into different subtypes depending on their molecular, functional, morphological, electrophysiological and anatomical characteristics. Recent single-cell RNA sequencing experiments on adult mouse tissue have helped characterize the profound molecular heterogeneity in the brain. For example, Saunders and colleagues analysed cells from nine brain areas and determined that these cells can be divided into 323 transcriptionally different neuronal subtypes [87].

The majority of these cells are generated from specific parts of the neural tube in exact temporal order during embryonic or early postnatal development. The final fates of mitotic progenitors and post-

mitotic precursors are dependent on both intrinsic and external molecular cues. In mitotic progenitors, cell fate is specified by extracellular morphogen gradients during neural tube patterning and by the expression of proneural genes. In early post-mitotic precursors, cell fate has not yet been terminally determined and can be altered. In post-mitotic neuronal development, neuron identity is specified by neuron-type selector genes and maintained by terminal selector genes in mature neurons.

2.2.1. Early development of the central nervous system: formation of the neural tube

All neurons of the CNS are derived from ectoderm that is generated during gastrulation. A central area of ectoderm differentiates further into neural ectoderm, which in turn gives rise to the neural plate. The neural plate is a flat layer of pseudostratified epithelial cells that are surrounded by the neural plate border (NPB) which isolates the neural plate from the lateral surface ectoderm (Figure 5). The NPB forms neural crest cells that give rise to multiple tissues including the peripheral nervous system. During development, the neural plate transforms into a cylindrical tube called the neural tube that is positioned in the midline of the embryo and gives rise to the CNS. To do this, the neural plate folds at distinct places starting anterior-posteriorly at E8.5, gradually involving the rest of the neural plate, such that, while in some parts the neural tube is closed, in other parts it is just being initiated [88]. In the future cranial region (anterior) of the neural tube, medial cells of the neural plate overlaying the mesodermal notochord go through morphological changes whereby the apical side constricts and becomes smaller than the basal side, thus forming the median hinge point (MHP) [89]. This apical constriction causes the NPB area to rise dorsally and gives the neural tube precursor a V-shape. A closed neural tube is achieved when the ends of the neural epithelium curve inwards and fuse together using cytoplasmic protrusions [90].

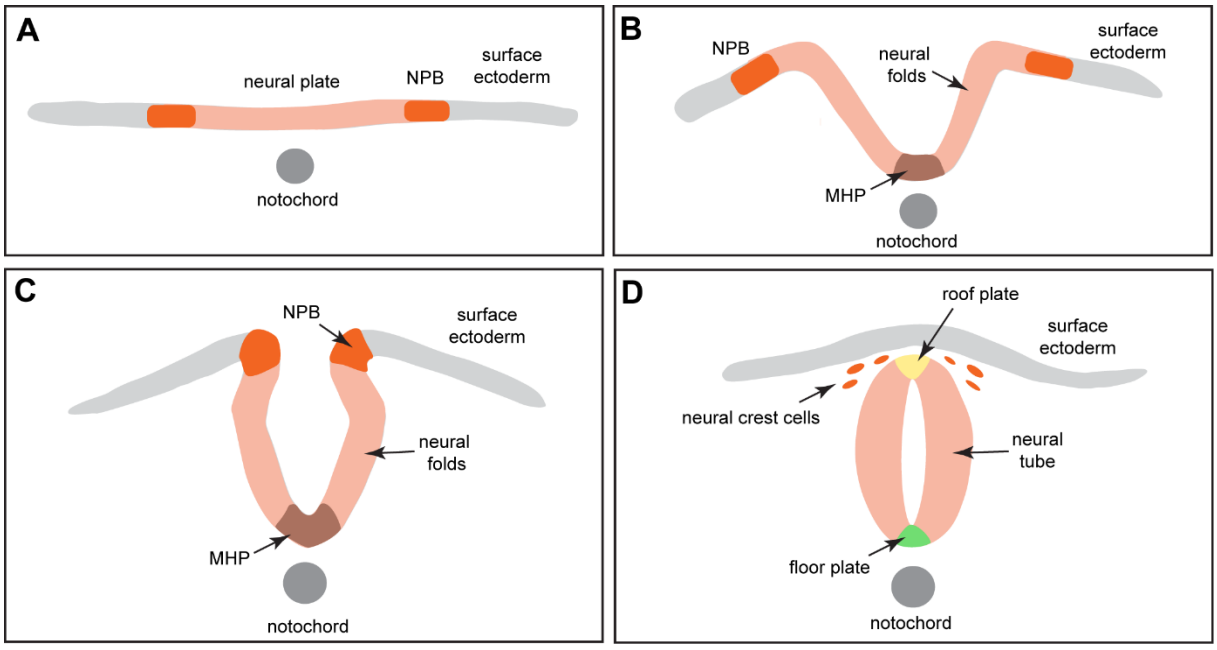

Figure 5. Formation of the neural tube. (A) A flat neural plate surrounded by NPB and surface ectoderm. (B-C) The lateral sides of neural plate rise dorsally bringing the NPB-s together. Neural folds fuse giving rise to the closed neural tube (D). Same time surface ectoderm fuses and NPB gives rise to neural crest cells that migrate away. MHP, median hinge point; NPB, neural plate border.

2.2.2. Patterning of the neural tube

2.2.2.1. Anterior-posterior patterning

The neural tube generates different brain regions on the anterior-posterior axis. Initially, the neural tube can be divided into three brain vesicles. They are the forebrain (prosencephalon), midbrain (mesencephalon) and hindbrain (rhombencephalon) (Figure 6A). Later, five brain vesicles can be distinguished, when the forebrain separates into the telencephalon and diencephalon, and the hindbrain separates into the metencephalon (pons) and the myelencephalon (medulla) (Figure 6B). The hindbrain is then further divided into individual transient developmental structures called rhombomeres. The hindbrain is anterior-posteriorly made up of rhombomeres 1-6 (r1-r6) whose borders are discernible, and pseudorhombomeres 7-11 (r7-r11) that lack any visible boundaries (Figure 6C) [91]. Moreover, some divide the r1 further into two segments: the isthmus (r0) that is located anteriorly, and the remaining r1 [92]. This division is based on gene expression differences between the two regions and differences in the nuclei they give rise to [93]. However, in this thesis I group these regions together and refer to them collectively as r1. In future studies, however, they should be considered as a two separate brainstem areas.

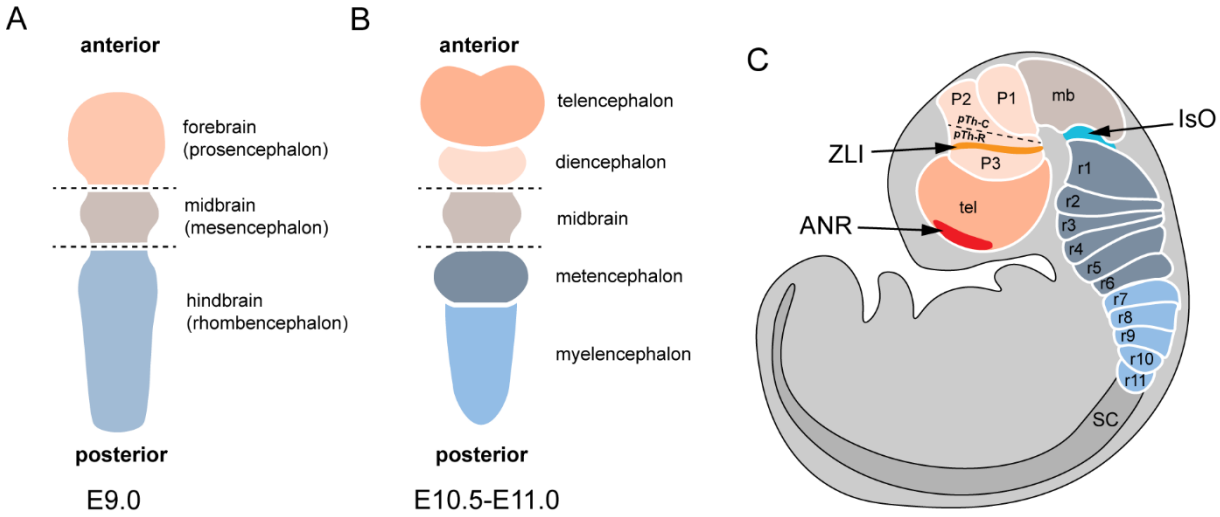

Figure 6. Anterior-posterior patterning of the neural tube. (A) Neural tube at the three vesicle stage. (B) Neural tube at the five vesicle stage. (C) The main regions of neural tube in developing mouse and the placement of three

organizing centres: ANR - anterior neural ridge, ZLI - zona limitans interthalamica and IsO - isthmic organizer. mb, midbrain; r, rhombomere; P1, pretectum; P2, thalamus; P3, prethalamus; pTh-C, thalamic progenitors, caudal; pTh-R, thalamic progenitors, rostral; SC, spinal cord; tel, telencephalon.

The rostral neuroepithelium is patterned anterior-posteriorly with the aid of secondary organizers positioned at the borders of different brain regions, while the hindbrain is divided into separate rhombomeres by the distinct expression of different Hox-family TFs in defined regions. The developing brain contains three secondary organizers: the anterior neural ridge (ANR) located most anteriorly in the telencephalon, the zona limitans interthalamica (ZLI) in the diencephalon, and the isthmic organizer (IsO) positioned at the midbrain-hindbrain boundary (Figure 6C). The ANR develops from the most anterior neural ectoderm region adjacent to the anterior surface ectoderm. It expresses *Fgf8* (*Fibroblast growth factor 8*) and the BMP (Bone Morphogenetic Protein) antagonists *Chordin* and *Noggin* that are required to inhibit BMP signalling, support cell survival, and induce forebrain-specific gene expression in the neighbouring rostral part of the neural plate [94, 95]. The telencephalon is adjacent rostrally to the diencephalon that can be divided anterior-posteriorly into three prosomeres (P): P3 – prethalamus, P2 – thalamus and P1 – pretectum. The ZLI is located between P3 and P2 and secretes SHH (Sonic hedgehog). SHH diffusion from the ZLI to adjacent regions is needed to create unique gene expression patterns on both sides of the diencephalon [96]. The SHH gradient in the thalamus also separates the developing thalamus into two domains: the rostral pTh-R (thalamic progenitors, rostral) which generates GABAergic neurons and the caudal pTH-C (thalamic progenitors, caudal) which generates glutamatergic neurons [97].

The third secondary organizer is the Isthmic Organiser (IsO). Before it forms, the rostral neural tube expresses *Otx2* (*Orthodenticle homeobox 2*), while the caudal region expresses *Gbx2* (*Gastrulation brain homeobox 2*). The expression of these two genes initially overlaps at the border of the midbrain and hindbrain. However, they soon start to repress one another and induce the expression of *Fgf8*. FGF signalling represses *Otx2* and activates *Gbx2* causing their expression to be restricted to midbrain and hindbrain, respectively while *Fgf8* expression is maintained in the most rostral *Gbx2* positive region of the hindbrain (isthmus) that is adjacent to the midbrain [98]. In addition to determining the midbrain and hindbrain boundary, the IsO is also required for the development of both the midbrain and the most rostral hindbrain r1 segment. Conditional inactivation of *Fgf8* in the midbrain and r1 causes the loss of the midbrain, isthmus and cerebellum in developing mouse embryos, while ectopic expression of *Fgf8* (bead) in the chick diencephalon can induce the ectopic formation of midbrain, isthmus and cerebellum-like tissue [99, 100]. FGF8 is responsible for maintaining midbrain and r1-specific expression of genes such as *En1*, *En2* (*Engrailed 1* and *2*), *Pax2* and *Pax5* (*Paired box 2* and *5*) that form FGF dependent gradients and are either required for the correct positioning of the IsO, the developmental regulation of the midbrain, r1, and cerebellar neurons [101-105]. For example, when midbrain dopaminergic

progenitors are unable to receive FGF-signalling, they acquire an ectopic gene expression pattern that is characteristic to anteriorly located diencephalon derived dopaminergic cells [106].

While the rostral brain is patterned by the help of secondary organizers, the hindbrain from r2 onwards and the spinal cord are patterned by *Hox* genes. The mouse genome contains 39 *Hox* genes that are divided into four clusters (A-D) positioned on different chromosomes. Each of these four chromosomes has 9-11 *Hox* genes (there are 13 different paralogues) that are located successively from 3′ to 5′ of the DNA. *Hox* genes encode for different TFs expressed anterior-posteriorly in the hindbrain and spinal cord, and divide the hindbrain into rhombomeres (except r1, where FGF signalling inhibits their expression) and the spinal cord into cervical, thoracic, lumbar, sacral and coccygeal segments [101, 107]. *Hox* genes positioned near the 3′ region of the DNA are expressed earlier in development and more anteriorly [108]. In addition to separating the hindbrain and spinal cord into different areas, *Hox* genes also control the positioning and development of several cell types in the hindbrain and spinal cord including oligodendrocytes, motor, sensory and serotonergic neurons [109].

2.2.2.2. Dorsal-ventral patterning

The neural tube is also patterned on its dorsal-ventral axis. After closure, it develops into a pseudostratified neuroepithelium containing neuronal progenitors. It includes two other areas: the floor plate located on the ventral midline derived from the MHP; and the roof plate formed during closure of the dorsal midline from the lateral edges of the neural plate (Figure 5). The floor plate, together with the adjacent notochord secretes SHH, while the roof plate and surface ectoderm secrete BMP signalling ligands. This creates SHH and BMP signalling gradients whereby the progenitors closest to the floor plate (most ventral) or the roof plate (most dorsal) receive the highest SHH or BMP signals, respectively, while progenitors in the centre of the neuroepithelium encounter lower morphogen concentrations. These concentration differences induce the expression of distinct TFs in precursors and divide the neural tube dorso-ventrally into several progenitor domains that give rise to different cell types.

Dorsal-ventral patterning is best characterized in the spinal cord. The ventricular zone is divided dorso-ventrally into eleven progenitor domains (Figure 7A). The dorsal spinal cord contains six progenitor domains (dP1-6) that give rise to dorsal interneurons (dI1-6) that are involved in the regulation of somatosensory information including nociception and proprioception [110]. The ventral spinal cord is divided into five progenitor domains (p0-p3, pMN) that generate different types of excitatory and inhibitory interneurons (V0-V3) or motoneurons (MN) that form the motor circuitry and coordinate contractions of skeletal muscles [111]. In the developing spinal cord, SHH activates the expression of homeodomain TFs *Nkx2-2*, *Nkx2-9* (*NK2 homeobox 2* and *9*), *Nkx6-1* and *Nkx6-2* (*NK6 homeobox 1* and *2*) in the most ventrally positioned progenitors, while BMP signalling is responsible for inducing the expression of *Pax6*, *Pax7* (*Paired box 6* and *7*) and *Msx1/2* (*Msh homeobox 1* and *2*) in dorsally located progenitors [112, 113]. A third signal that also influences patterning originates from the

paraxial mesoderm located lateral to the neural tube. Retinoid signalling from the paraxial mesoderm induces expression of *Dbx1* and *Dbx2* (*Developing brain homeobox 1* and *2*) in progenitors located medially [114]. SHH induced NKX2-2 and NKX6-1 represses the expression of these and dorsally expressed genes, whereas BMP signalling with dorsal TFs represses medially and ventrally expressed genes [115, 116]. Transplantation of *Shh* positive notochord or floor plate to the lateral side of the neural tube causes the development of ectopic motor neurons and interneurons from the adjacent neural tube that are normally seen in the most ventral neural tube [117]. Homeodomain TFs induce the expression of several proneural bHLH TFs (discussed below) required to divide the ventricular zone into smaller progenitor domains and specify neuronal subtypes [118].

The dorsal-ventral patterning of r1 has not been described in similar detail. As is the case with many other brain areas, it is divided by the sulcus limitans into the ventrally located basal plate and the dorsally located alar plate and contains several progenitor domains which give rise to different cell types (Figure 7B). The most ventral progenitors in the basal plate of r1, near the floor plate, generate serotonergic neurons that develop into to the dorsal raphe (DR) and a portion of the median raphe [119]. Likewise, in the spinal cord, SHH signalling in the floor plate activates *Nkx2-2* and *Nkx6-1* expression in ventral neighbouring progenitors that have been shown to be important for serotonergic neuron specification [120, 121]. *Nkx6-1* expression extends more dorsally than *Nkx2-2* and includes Nkx6-1^{+} progenitor domain that produces GABAergic and glutamatergic neurons that contribute to various nuclei in the ventral midbrain and anterior hindbrain including the interpeduncular nucleus (IPN) and substantia nigra pars reticulata (SNpr) [122-124]. A small progenitor domain in the most dorsal part of the basal plate (adjacent to the alar plate) has been shown to express *Dbx1* and *Dbx2*, where expression of *Dbx1* is SHH-dependent unlike in the spinal cord [121, 122]. The dorsally located alar plate is divided into three domains: the rhombic lip, and the intermediate and ventral domains. All progenitors in the alar plate express *Pax7*, while the rhombic lip also expresses *Msx1* and *Msx2*, possibly also induced by BMP signalling from the roof plate similar to the spinal cord [122, 125]. These three domains additionally express different bHLH TFs and give rise to different nuclei. The rhombic lip expresses *Atoh1* (*Atonal bHLH transcription factor 1*, also known as *Math1*) and *Olig3* (*Oligodendrocyte transcription factor 3*), and gives rise to glutamatergic granule cells in the cerebellum, as well as to neurons that migrate ventrally into the basal plate and constitute several nuclei in the rostral hindbrain including the lateral parabrachial nucleus (LPN), laterodorsal tegmental nucleus (LDTg) and pedunculopontine tegmental nucleus (PPTg) [126]. The intermediate domain expresses *Ascl1* (*Achaete-scute family bHLH transcription factor 1*, also known as *Mash1*) (expressed also in the basal plate), *Neurog2* (*Neurogenin 2*) and *Ptf1a* (*Pancreas specific transcription factor, 1a*), and gives rise to

GABAergic neurons that populate the cerebellum, whereas the ventral domain expresses *Ascl1* (weakly), *Neurog1* and *Neurog2*, and generates neurons of the locus coeruleus and IPN [122, 125, 127].

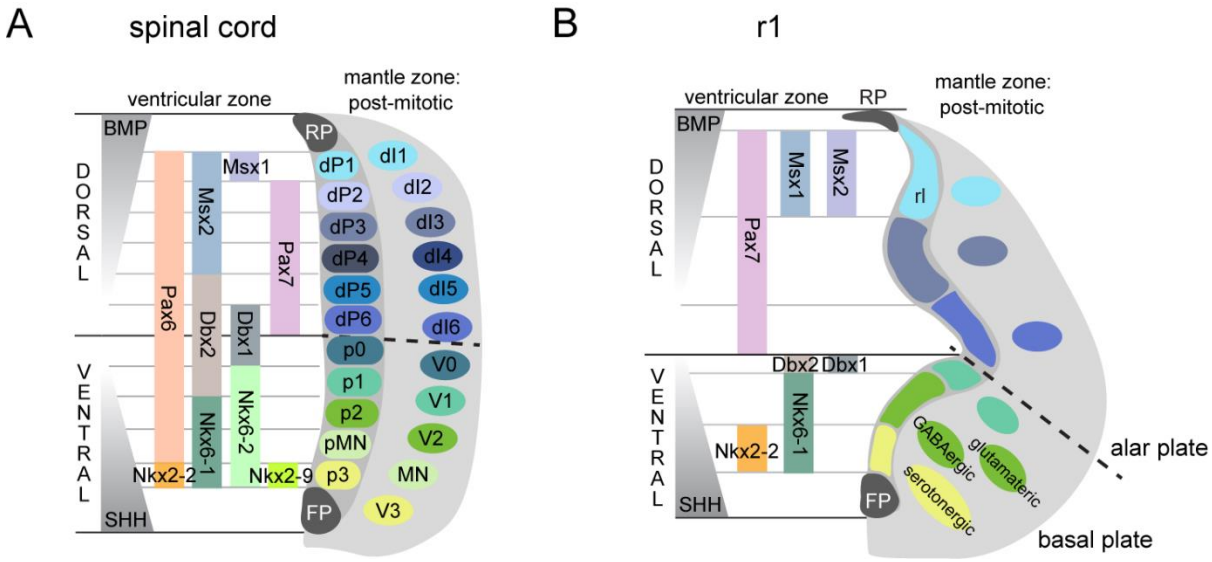

Figure 7. Dorsal-ventral patterning of the spinal cord and r1. (A) The ventricular zone of spinal cord is divided into eleven progenitor domains that express different TFs involved in the pattering of spinal cord and give rise to distinct post-mitotic cell types. (B) Similarly, the ventricular zone of r1 can be divided into at least six progenitor domains. FP, floor plate; RP, roof plate; rl, rhombic lip; dP, dorsal progenitor; p, progenitor; MN, motoneuron; dI, dorsal interneuron; V, ventral interneuron.

2.2.3. Determination of cell fate

2.2.3.1. Proneural genes control neurogenesis and cell fate

After the neural tube has been patterned by homeodomain TFs, progenitors begin expressing proneural genes that encode bHLH class TFs. Vertebrate genomes contain 23 proneural genes that are separated into distinct families based on similarities in their bHLH domain [128]. Proneural genes bind the E-box and mostly operate as a transcriptional activators promoting neurogenesis, cell-cycle exit, differentiation, and neuronal specification [4, 128]. In progenitor cells, proneural genes are involved in a process called lateral inhibition, whereby one cell prevents an adjacent one from exiting the cell-cycle, and in doing so prevents the premature depletion of the progenitor pool. Proneural genes also upregulate the expression of Notch ligands, such as *Dll1* (*Delta-like 1*) which activate Notch signalling in neighbouring cells. Notch signalling upregulates Notch effector genes such as TF *Hes1* (*Hes family bHLH transcription factor 1*), which in turn repress expression of proneural genes and *Dll1*. This signalling between cells takes place repeatedly, causing expression levels of *Hes1*, *Dll1* and proneural

genes to oscillate every 2-3 hours between low and high (when *Hes1* expression is high, that of *Dll1* and proneural genes is low as HES1 supresses their expression) [129]. This oscillation is required for normal cell-cycle progression and the proliferation of neural progenitor cells, while sustained expression of proneural genes in progenitors promotes cell-cycle exit and differentiation [130]. Different proneural genes are responsible for the differentiation of distinct neuronal and glial cell types. For example, *Ascl1* facilitates differentiation of GABAergic and serotonergic neurons, *Neurog2* promotes development of glutamatergic and dopaminergic neurons, while *Olig1* and *Olig2* are required for the differentiation of motor neurons and oligodendrocytes [131-136].

2.2.3.2. Post-mitotic cell fate determination by selector genes

After cell-cycle exit, neuronal precursors leave the ventricular zone and initiate the differentiation process by activating genes fundamental to the function of specific neuronal subtypes. Before cell-cycle exit or soon thereafter, precursors start expressing neuron-type selector genes (post-mitotic TFs) responsible for determining neuronal identities by controlling multiple cell fate choices and directing precursors to differentiate towards a specific lineage [5, 6]. The loss of neuron-type selector genes causes precursors to differentiate into alternative lineages. For example, in the mouse, *Gata2* and *Tal2* act as post-mitotic neuron-type selector genes in midbrain development where they promote a GABAergic fate. In the absence of either of these, precursors acquire a glutamatergic identity [9, 10]. Additionally, TFs *Tlx1* and *Tlx3* (*T-cell leukaemia, homeobox 1* and *3*) have been suggested to operate as neuron-type selector genes in dorsal spinal cord precursors where they induce glutamatergic and repress GABAergic fates [8].

Terminal selector genes are those TFs whose expression continues in mature neurons, where they maintain specific neuronal identity by directly binding regulatory sequences of terminal differentiation genes. Terminal differentiation genes are those required to attain characteristic features of unique neuronal identity, such as enzymes and proteins needed to synthetize, metabolise or transport specific neurotransmitters, receptors and ion channels. Inactivation of terminal selector genes in mature neurons causes the loss of cell identity, while pan-neuronal features (the presence of axons, dendrites, synapses) are retained [7, 137]. The role of terminal selector genes in the mouse have been better studied than neuron-type selector genes. Several of the former have been identified. Examples include: *Nr4a2* (*Nuclear receptor subfamily 4, group A, member 2*, also known as *Nurr1*), *Pitx3* (*Paired-like homeodomain transcription factor 3*) in dopaminergic neurons, and *Pet1* (*FEV transcription factor, ETS family member,* also known as *Fev*), *Lmx1b* (*LIM homeobox transcription factor 1 beta*) and *Gata3* in serotonergic neurons [138-141].

2.3. Function of GATA TFs in the development of GABAergic and serotonergic neurons

2.3.1. GATA TFs and their co-regulators in the central nervous system

Of the GATA TFs, *Gata2*, *Gata3*, *Gata4* and *Gata6* are expressed in the CNS. *Gata4* and *Gata6* are expressed in developing and adult neurons, and astrocytes. In astrocytes they act as a tumour suppressor genes in astrocytoma (astrocytic tumour), but their function in neurons has not yet been determined [16, 142-144]. *Gata2* and *Gata3* as well as their co-regulators *Tal1* and *Tal2* are expressed in specific regions of the embryonic CNS, where they are important for neuronal differentiation. In the developing diencephalon, midbrain, r1 and spinal cord they are expressed in GABAergic precursors and are required for GABAergic neuron development from these areas (discussed in more detail below) [9, 10, 132, 145, 146]. Additionally, GATA2 and GATA3 are also expressed in immature serotonergic precursors in the hindbrain and are needed for appropriate development (discussed in more detail below) [10, 120, 147].

Furthermore, GATA cofactors *Zfpm1* and *Zfpm2* are also expressed in the developing CNS. Expression of *Zfpm1* has not been well characterized in CNS development. It has only been detected at E12.5 in the midbrain and in spinal cord V2b interneurons between E9.5-E13.5. *Zfpm2* is expressed from E10.5 onwards in the spinal cord and midbrain, and from E13.5 in the basal ganglia, hypothalamus and hindbrain [71, 72, 118]. Their function in the CNS has not yet been completely defined. However, it has been shown that ZFPM2 controls the differentiation of one subtype of corticothalamic projection neurons (CThPN) in the cortex by co-operating with GATA2 and GATA4 to repress the expression of *Bcl11b* (*B-cell leukaemia/lymphoma 11B*, also known as *Ctip2*) [148].

2.3.2. GABAergic neurons

GABAergic neurons are inhibitory neurons that utilise gamma-aminobutyric acid (GABA) as a neurotransmitter. GABA is synthesized from glutamate in GABAergic cells by two glutamic acid decarboxylases: GAD1 (Glutamate decarboxylase 1, also known as GAD67) and GAD2 (Glutamate decarboxylase 2, also known as GAD65). Although both are expressed in the brain, GAD1 is expressed at higher levels and throughout the cell, while GAD2 is mostly found in nerve endings [149]. GABA is transported into synaptic vesicles by the vesicular inhibitory amino acid transporter VIAAT [150] and taken up from the synaptic cleft by GABA Transporters 1-3 (GAT1-3). GAT1 and GAT3 are extensively expressed in the brain by pre-and postsynaptic neurons as well as astrocytes [151]. Imported GABA is then reused or metabolized into the tricarboxylic acid cycle intermediate succinate.

2.3.2.1. Development of GABAergic neurons

GABAergic neurons develop throughout the neural tube and give rise to an abundant number of diverse nuclei in the adult brain. The development of GABAergic neurons is regulated by different proneural genes and post-mitotic TFs in different parts of the neural tube. GATA TFs and their co-regulators have been shown to be expressed in post-mitotic GABAergic precursors in the diencephalon, midbrain, r1 and spinal cord, where some are also required for correct development.

The early development of mitotic GABAergic progenitors is well studied in the diencephalon and midbrain. In these areas, GABAergic development is under the control of the proneural gene *Ascl1* and the bHLH gene *Helt* (*Hairy and enhancer of split-related protein*). Both are expressed in GABAergic progenitors located in the midbrain and are also found in diencephalon regions pTh-R and P1. In the midbrain, HELT is required for the specification of GABAergic over glutamatergic identity. It promotes expression of genes needed for the differentiation of GABAergic neurons such as *Gata2* and *Gad1* and simultaneously represses proneural genes *Neurog1* and *Neurog2* that specify glutamatergic identity [9, 136]. ASCL1 facilitates cell-cycle exit in the ventral midbrain and is required for the development of GABAergic neurons in the dorsal midbrain. Unlike HELT, inactivation of *Ascl1* does not cause a cell fate switch from GABAergic to glutamatergic [133]. Both ASCL1 and HELT are necessary for the development of GABAergic neurons from the diencephalon. In the P1, ASCL1 promotes a GABAergic over glutamatergic fate. Inactivation of *Ascl1* causes the loss of P1 GABAergic neurons, induces the ectopic expression of *Neurog2* and the development of glutamatergic neurons [132]. In the pTh-R, ASCL1 and HELT act together to supress the expression of rostral P3 markers such as *Dlx2*, *Dlx5* (*Distal-less homeobox 2* and *5*) and *Arx* (*Aristaless related homeobox*). In *Ascl1* and *Helt* double mutants, pTh-R GABAergic neurons differentiate into P3 GABAergic neurons instead, suggesting that there they are needed to determine GABAergic neuron subtype [152]. *Ascl1* is also expressed in ventral r1, but is not needed there for the development GABAergic neurons [133].

After cell-cycle exit, GABAergic development is under the control of post-mitotic TFs that act as neuron-type selector genes to determine GABAergic identity or specify GABAergic subtype. *Gata2*, *Gata3* and *Tal1*, *Tal2* are all expressed in post-mitotic GABAergic precursors in the diencephalon (P1 and pTH-R), midbrain, r1 and spinal cord. In the midbrain, *Gata2* and *Tal2* operate as a neuron-type selector genes by specifying GABAergic fate over glutamatergic. Inactivation of *Gata2* or *Tal2* causes complete loss of midbrain GABAergic cells and their marker genes *Gata3*, *Tal1* and *Six3* (*Sine oculis-related homeobox 3*), while ectopic glutamatergic neurons are generated instead [9, 10]. Although TAL1 alone is not required for the development of midbrain GABAergic cells, it works redundantly with TAL2 to specify a certain subset of GABAergic cells in the ventral midbrain [10]. In the diencephalon, *Gata2* acts similarly as a neuron-type selector gene in the P1 region, while in the pTh-R, GATA2 does not determine GABAergic over glutamatergic fate but is needed to acquire a GABAergic pTh-R subtype.

In *Gata2* conditional mouse mutants, P1 precursors differentiate into glutamatergic neurons and the pTh-R starts to ectopically express markers specific to P3 GABAergic neurons [132].

In the spinal cord, ventral p2 progenitors give rise to $Gata2^{+}Gata3^{+}$ inhibitory V2b interneurons (GABAergic) and to $Vsx2^{+}$ (*Visual system homeobox 2*) excitatory V2a interneurons (glutamatergic). The fate choice between these two cell types is determined by GATA2, TAL1, LHX3 (LIM homeobox protein 3) and Notch signalling. p2 progenitors express *Gata2*, *Lhx3* and, as a result of the Notch signalling, begin to express different levels of the Notch receptor *Notch1* and its ligand *Dll4* on their cell surface. Cells that receive a greater amount of Notch signalling ($Notch1^{+}$) up-regulate *Tal1* expression and start to differentiate towards a GABAergic V2b lineage, while other cells ($Dll4^{+}$) become glutamatergic V2a neurons [153]. This suggests that *Tal1* might act as neuron-type selector gene in the spinal cord. In *Tal1* KO embryos, V2b cells are absent, while the number of V2a neurons present are higher. Additionally, ectopic expression of *Tal1* in the spinal cord is sufficient to promote the differentiation of V2b cells and at the same time inhibit a V2a cell fate [146, 154]. Unlike TAL1, GATA2 is required in progenitors for the normal development of both interneuron types, inhibits their differentiation into a motor neuron lineage, and promotes a V2b lineage over V2a lineage fate. In *Gata2* KO embryos, both V2a and V2b cells are significantly reduced, while ectopic expression of *Gata2* in electroporated spinal cord cells induces the expression of V2b-specific markers and inhibits the expression of motor neuron and V2a markers [145, 155]. Interestingly, TAL1 and GATA2 co-operate to facilitate the differentiation of V2b interneurons by regulating gene expression in precursors. TAL1 and GATA2 are assembled via LMO4 (LIM domain only 4), into the protein complex TAL1/TCF3/LMO4/LDB1/GATA2 that binds enhancers in the *Gata2* and *Gata3* loci and up-regulates their expression [146]. Recently, *Zfpm1* was also shown to be expressed in developing V2b interneurons suggesting that it might also be involved together in their differentiation [118].

Compared to other brain regions, not much is known about GABAergic development in r1. What is known is that GATA2 and GATA3 alone are not required [9], while TAL1 does seems to play an important role in their development. In *Tal1* conditional mouse mutants, GABAergic neurons are absent in r1 at early embryonic stages and the r1-derived GABAergic nucleus pSNpr is lost at late prenatal stages. However, the exact mechanism of TAL1 function in r1 has yet to be uncovered [123].

2.3.2.2. GABAergic nuclei in the anterior brainstem

The brainstem area of the CNS is comprised of the midbrain and hindbrain (pons and medulla). It contains many important nuclei involved in the regulation of various essential body functions including respiration, blood pressure, locomotion, sleep/wakefulness, emotion, and motivated behaviour [156-161]. Several GABAergic nuclei are located in anterior brainstem. Yet while their functionality has been the focus of much investigation, little is known about their developmental origin, cell subtype composition and corresponding identifying markers. The following provides an overview of some of these nuclei, specifically the SNpr, IPN, LDTg, ventral and dorsal tegmental nuclei (VTg and DTg) and rostromedial tegmental nucleus (RMTg) (Figure 8). It should be noted that many of the GABAergic and glutamatergic neurons in these nuclei are involved in the control of dopaminergic and serotonergic systems.

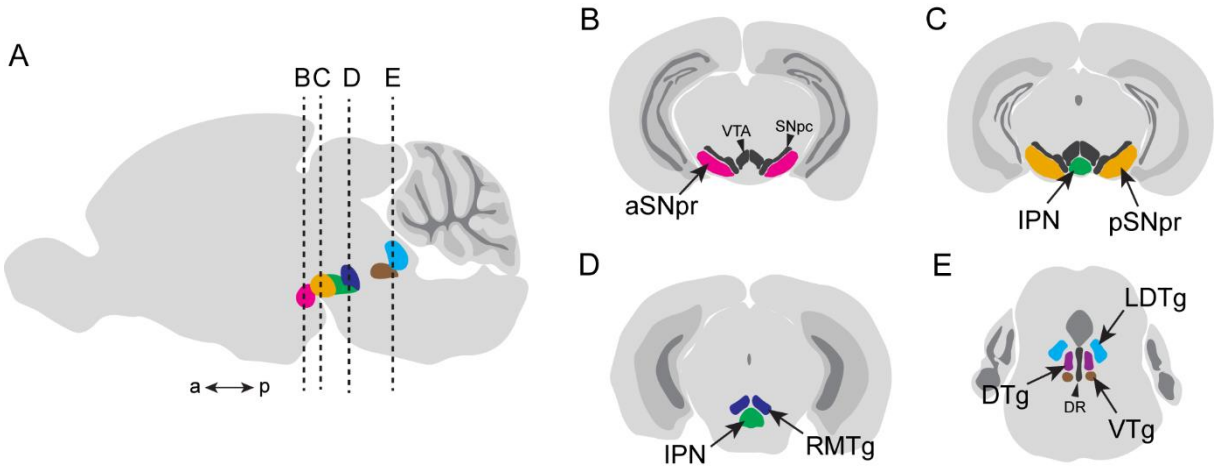

Figure 8. Selected nuclei containing abundant GABAergic neurons in the anterior brainstem. (A-E) Sagittal (A) and coronal views (B-E) of adult mouse brain showing the positions of different GABAergic nuclei in the anterior brainstem. VTA, SNpc, and DR are shown has a reference. aSNpr, anterior substantia nigra pars reticulata; DR, dorsal raphe; DTg, dorsal tegmental nucleus; IPN, interpeduncular nucleus; LDTg, laterodorsal tegmental nucleus; pSNpr, posterior substantia nigra pars reticulata; RMTg, rostromedial tegmental nucleus; SNpc, substantia nigra pars compacta; VTA, ventral tegmental area; VTg, ventral tegmental nucleus; a, anterior; p, posterior.

1) Substantia nigra pars reticulata

The substantia nigra is divided dorso-ventrally into two parts. The SNpr, located in the ventral midbrain, is an output nucleus of the basal ganglia and controls voluntary movement and motivated behaviour. The substantia nigra pars compacta (SNpc) is positioned dorsally and is composed of dopaminergic neurons. The SNpr contains GABAergic projection neurons [162], belongs to the basal ganglia system, and receives both direct and indirect innervation from the striatum of the rostral basal ganglia [163]. It sends out efferent fibres from the basal ganglia to the thalamus, superior colliculus and PPTg, and regulates movement execution [164-166]. When SNpr GABAergic neurons are inactivated, inhibition of these structures is decreased, thus allowing movement to occur [167]. Collateral innervation of the SNpc by SNpr efferent projections inhibits dopaminergic neurons [168]. As a modulator of the SNpc, the SNpr has also been implicated in reward learning, cognition, mood, and alcohol withdrawal [169-171]. It is composed of neurons that have different developmental origins. Neurons in the posterior part (pSNpr) originate from r1 and migrate to the ventral midbrain while neurons in the anterior part (aSNpr) have been suggested to be derived from the midbrain or diencephalon [123, 172]. At present, the potentially unique properties and functions of these subtypes remain poorly understood and thus warrant further study.

2) Rostromedial tegmental nucleus

The RMTg is a GABAergic nucleus located in the ventral midbrain. Its rostral part is positioned above the caudal IPN and is medial to the ventral tegmental area (VTA). The caudal region extends caudally beyond the VTA, reflecting its additional name “tail of the VTA.” The RMTg is moderately innervated by numerous brain structures but receives dense excitatory innervation from the lateral habenula, forming circuitry that is implicated in aversion and avoidance behaviour [173]. In turn, RMTg projects to and inhibits the VTA and SNpc [174]. For this reason, RMTg is an important regulator of the midbrain dopaminergic system that is involved in the control of addiction, movement and reward [175-177]. Neurons in the RMTg activate the expression of the immediate early genes *c-Fos* (*FBJ osteosarcoma oncogene*) and *FosB* (*FBJ osteosarcoma oncogene B*) in response to acute or chronic administration of psychostimulants such as cocaine [174, 178]. Consequently, *c-Fos* expression has been used to distinguish the RMTg, other specific markers of remain scarce. The developmental program of RMTg remains largely uncharacterized.

3) Ventral and dorsal tegmental nuclei

The VTg and DTg are two GABAergic nuclei located in the anterior hindbrain. The VTg is positioned rostrally below the DR, while the DTg is adjacent to it but located more caudally between

the DR and LDTg. The VTg contains neurons positive for the calcium-binding proteins Parvalbumin and Calbindin, innervates the medial mammillary nucleus, and has been shown to regulate theta rhythm and spatial memory. Neurons in the DTg also express Parvalbumin but innervate the lateral part of the mammillary nucleus and are involved in the regulation of the head-direction system [179, 180]. As is the case with the RMTg, the developmental program of these two nuclei has yet to be determined.

4) Laterodorsal tegmental nucleus

The LDTg is located bilaterally in the anterior hindbrain close to the 4th ventricle, between the DTg (medially) and the locus coeruleus (laterally). It is a heterogeneous nucleus, containing GABAergic, glutamatergic and cholinergic neurons that are intermingled yet distributed unevenly within the nucleus. Glutamatergic and GABAergic neurons are located more in the rostral and caudal LDTg respectively, while cholinergic neurons are found to a greater extent in the medial LDTg [181]. LDTg neurons receive dense afferents from the lateral habenula, lateral hypothalamus and periaqueductal gray, and lower innervation from the medial prefrontal cortex, substantia nigra, superior colliculus and DR [182]. It also sends efferent fibres to numerous brain areas including the medial prefrontal cortex, thalamus, lateral hypothalamus, superior colliculus, lateral habenula, SNpc, VTA and IPN [183]. LDTg has been implicated in regulation of reward and sleep. Excitation of LDTg glutamatergic and cholinergic innervation to the VTA facilitates reward [184], while activation of cholinergic neurons promotes rapid eye movement (REM) sleep [185]. The LDTg has been shown to develop from r1 and also contains cells derived from *Atoh1*-positive progenitors in the rhombic lip [126].

5) Interpeduncular nucleus

The IPN is located in the midline of the ventral midbrain just below the VTA and RMTg, and extends caudally to the median raphe nucleus. In rodents, it is divided into three unpaired and four paired (bilaterally situated) subnuclei, while in the chicken it is divided into 11 subnuclei based on gene expression traits and developmental origin [122, 186]. The majority of IPN neurons are GABAergic, although rostral subnuclei also contain Vglut2^{+} (*Vesicular glutamate transporter 2*, also known as *Slc17a6*) positive glutamatergic neurons, and caudal subdivisions contain some Vglut3^{+} (*Vesicular glutamate transporter 3*, also known as *Slc17a8*) glutamatergic neurons [187]. In addition, IPN subnuclei have distinct afferent and efferent projections [187]. The most important and substantial IPN afferent is the medial habenula. Other significant IPN projection targets include the septum, hippocampus, dorsal and median raphe, nucleus incertus and LDTg [188]. The medial habenula and IPN circuitry has been shown to be involved in causing the aversive effects of nicotine withdrawal (anxiety, sweating) after chronic nicotine use and has also been implicated in the regulation of anxiety, fear and depression [189, 190]. As of yet, the development of IPN neurons has not been thoroughly studied. In

the chicken, it has been shown that it is composed of neurons derived from different areas of r1. Nkx6-1$^+$ progenitors and Otp$^+$ (*Orthopedia homeobox*) post-mitotic cells in the basal plate of r1, as well as Pax7$^+$ progenitors from the alar plate of r1 that migrate tangentially to the ventral midbrain [122]. In mice, it has been determined that the specification and positioning of IPN cells is dependent on *Shh* expression originating from the floor plate [191].

2.3.3. Serotonergic neurons

Serotonergic neurons utilise serotonin (5-hydroxytryptamine, 5-HT) that acts as either an excitatory or inhibitory neurotransmitter, depending on the receptor it binds [192, 193]. Serotonin is synthesised from the amino acid tryptophan which is first hydroxylated to 5-hydroxytryptophan (5-HTP) by tryptophan hydroxylase and subsequently decarboxylated to serotonin by AADC (Aromatic L-amino acid decarboxylase). There are two tryptophan hydroxylases: TPH1 (Tryptophan hydroxylase 1) and TPH2 (Tryptophan hydroxylase 2). *Tph1* is expressed mostly in the pineal gland and gastrointestinal tract, whereas *Tph2* is brain-specific and is expressed by serotonergic neurons [194, 195]. Serotonin is accumulated into synaptic vesicles by the vesicular monoamine transporter 2 (VMAT2). Some serotonergic neurons express additionally the vesicular glutamate transporter VGLUT3 and are able to use glutamate as a second neurotransmitter [196]. Released extracellular serotonin is taken up again on presynaptic neurons by the serotonin transporter SERT (Sodium-dependent serotonin transporter, also known as SLC6A4). Internalized serotonin is then either transported again into synaptic vesicles and rereleased, or is degraded by monoamine oxidases.

2.3.3.1. Development of serotonergic neurons

Unlike GABAergic neurons, serotonergic neurons develop solely from the hindbrain. They are divided into rostral (r1-r3) and caudal (r5-r11) clusters based on their developmental origin [197, 198]. The rostral cluster gives rise to four serotonergic nuclei in the anterior hindbrain: the DR, median raphe, caudal linear nucleus and supralemniscal raphe. The caudal cluster generates an additional four nuclei: the raphe magnus, raphe pallidus, raphe obscurus and supragenual raphe nucleus [197, 199].
Serotonergic neurons develop from a small progenitor domain in the ventral hindbrain adjacent to the floor plate. These progenitors express TFs *Nkx2-2, Nkx2-9, Nkx6-1* and *Phox2b* (*Paired like homeobox 2B*), and give rise to *Phox2b, Isl1* and *Isl2* (*ISL1 transcription factor, LIM/homeodomain 1* and *2*, also known as *Islet-1* and *Islet-2*) positive motor neurons at E9.0-E9.5. Starting from E10.5, *Phox2b* and *Nkx2-9* expression is restricted to the dorsal part of the Nkx2-2$^+$ domain, dividing it into two regions. The dorsally located Nkx2-2$^+$Nkx6-1$^+$Phox2b$^+$Nkx2-9$^+$ domain continues to produce motor neurons, while the ventrally located Nkx2-2$^+$Nkx6-1$^+$ domain starts to generate serotonergic neurons [198]. In progenitors, the homeodomain TF PHOX2B induces the expression of proneural TFs that are essential

for cell-cycle exit, whereas in post-mitotic cells it determines motor neuron identity and is required for their differentiation [200, 201]. This temporal switch from motor to serotonergic neuron generation does not take place in two locations in the hindbrain: r1 and r4. In r1, it is thought that motor neuron development does not precede the development of serotonergic neurons and, therefore, they exit the cell-cycle approximately at E9.5 – one day earlier than the rest of hindbrain serotonergic neurons. Still, the origin of motor neurons that give rise to the trochlear nucleus remains controversial with r1 being suggested as their birthplace [93, 202]. Furthermore, progenitors in r4 generate only motor neurons and inactivation of *Phox2b* causes ectopic production of serotonergic neurons [198]. The temporal switch and spatial differences of motor neuron versus serotonergic development is determined by the TF FOXA2 (Forkhead box A2). *Foxa2* is expressed in r1 from E9.5 and r2-r11 (except r4) from E10.5, where it represses *Phox2b* and therefore inhibits motor development. Additionally, its expression is needed in progenitors to acquire serotonergic identity after *Phox2b* downregulation in the Nkx2-2^{+}Nkx6-1^{+} domain [203]. This progenitor region also expresses the proneural gene *Ascl1* that is required for serotonergic neurogenesis. In *Ascl1* mutants, progenitors in the ventricular zone fail to produce post-mitotic precursors and development of serotonergic neurons is impaired [131]. ASCL1 also directly activates the expression of the zinc-finger TF *Insm1* (*Insulinoma-associated 1*) during cell-cycle exit [204].

In post-mitotic precursors, INSM1 initiates a transcriptional cascade required for the differentiation of serotonergic neurons. It is necessary for the expression of *Gata2* and *Lmx1b* in early precursors. In both *Gata2* and *Lmx1b* mutants, all serotonergic neurons are absent [9, 120, 205]. Misexpression of *Gata2* causes the ectopic differentiation of serotonergic neurons in the hindbrain and is sufficient to induce *Lmx1b* expression, whereas ectopic induction of *Lmx1b* is not capable of inducing serotonergic differentiation [120]. This suggests that GATA2 is positioned at the top of a TF hierarchy that controls serotonergic cell differentiation and identity. GATA2 directly binds the regulatory region of the ETS-family TF *Pet1* and activates its expression in precursors [206]. PET1 has binding sites at regulatory regions of multiple serotonergic-specific genes necessary for the synthesis and transport of serotonin, and is responsible for their activation [207, 208]. As such, PET1 is necessary in precursors to acquire serotonergic identity [209]. Additionally, *Gata3* is expressed in post-mitotic serotonergic precursors, where it is controlled by GATA2 [120]. However, *Gata3* has been shown to be important only for the development of the caudal cluster (r5-r11) of serotonergic neurons. In *Gata3* KO embryos, caudal hindbrain serotonergic nuclei contain fewer serotonergic neurons, while rostral nuclei (derived from r1-r3) exhibit normal numbers [131, 147].

After the initiation of *Pet1*, *Lmx1b* and *Gata3* expression in precursors, it is maintained continuously in serotonergic neurons throughout development and into adulthood. In late embryonic development, PET1 is necessary for the maturation of serotonergic neurons by activating genes involved in migration, axon guidance and synaptic transmission [208]. In established serotonergic neurons *Pet1*

acts as terminal selector gene and preserves serotonergic identity by maintaining expression of a unique set of cell type specific genes [140]. Experiments using *Lmx1b* conditional mouse mutants, where *Lmx1b* was inactivated in *Pet1*$^{+}$ cells during embryonic stages showed that it is not only required for the generation and survival of serotonergic neurons, but also for *Pet1* expression and phenotype maintenance [141]. Inactivation of *Lmx1b* in adult neurons impairs expression of *Tph2, Sert* and *Vmat2* but not *Pet1* [210]. Similarly, GATA3 is essential to preserve the serotonergic identity. Conditional inactivation of *Gata3* in Pet1^{+} neurons demonstrated that GATA3 is not required for the survival of serotonergic neurons, as normal numbers of neurons derived from Pet1^{+} cells were found in the adult DR. However, inactivation of *Gata3* in this region caused a decrease in the expression of some serotonergic specific genes in mature neurons including *Tph2, Aadc, Sert* and *Vmat2,* suggesting that it may also function as a terminal selector gene [140].

2.3.3.2. Dorsal raphe

1) Subregions and heterogeneity of dorsal raphe

The DR is the largest serotonergic nucleus originating exclusively from r1 [119]. It is located dorsally in the midline of anterior hindbrain, although a narrow rostral region also extends to midbrain [197]. Classically, it has been divided anatomically and functionally into eight different subregions: (1) rostral, (2) dorsal (DRD), (3) ventral (DRV), (4) core of DRD (DRDc), (5) shell of DRD (DRDsh), (6) ventrolateral part of DR (DRVL), (7) caudal DR (DRC) and (8) interfascicular part of DR (DRI) (Figure 9) [211]. The rostral DR subregion is positioned most anteriorly, between the midbrain oculomotor nuclei. Posteriorly from it are situated the DRD and DRV subregions. The DRD is split further into two areas: the DRDc that is surrounded by neurons that comprise the DRDsh, and the DRVL that is positioned laterally on both sides of the caudal DRD (also referred to as the "lateral wings" of the DR). Most posteriorly located DR subregions are the DRC (located dorsally on the midline) and DRI which is located ventrally below the DRC. The DR is molecularly heterogeneous and composed of various cell types. Recently, Huang and colleagues characterized its heterogeneity in the adult mouse using single-cell RNA sequencing [212]. They showed that in addition to serotonergic neurons, it also contains dopaminergic, GABAergic, glutamatergic and peptidergic neurons. Moreover, they were able to identify five molecularly defined subtypes of serotonergic neurons located in distinct areas of the DR.

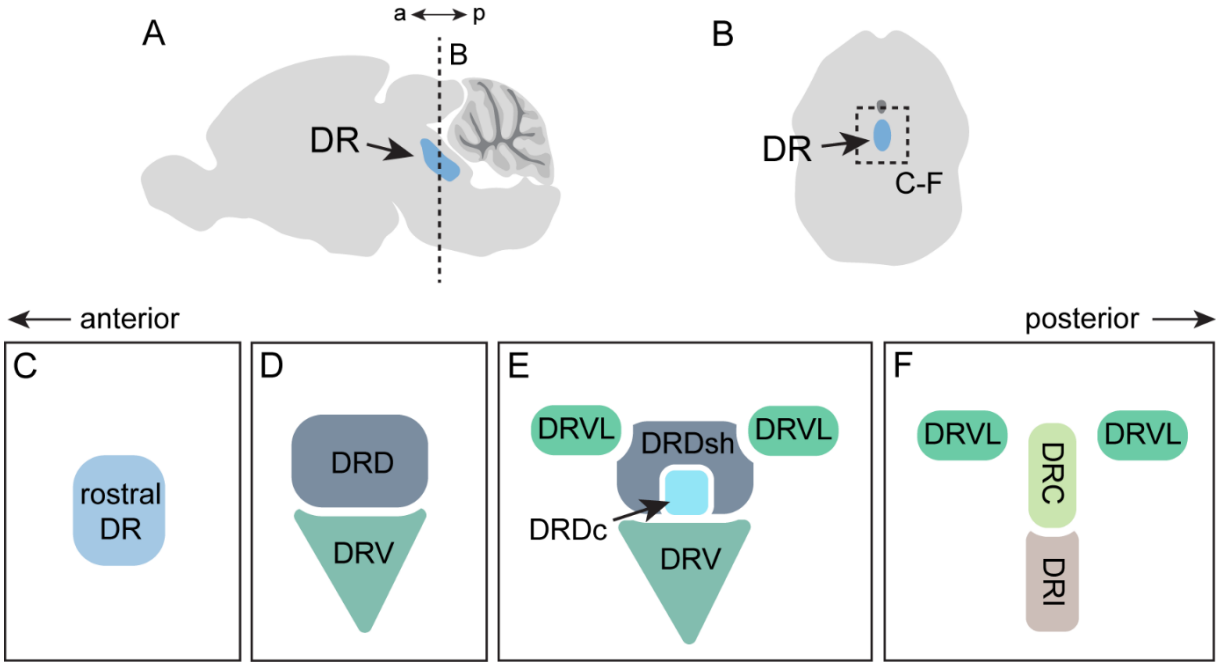

Figure 9. DR can be divided into several subregions. (A-B) Schematic presentation of sagittal (A) and coronal (B) section of adult mouse brain showing DR location. (C-F) DR subregions at different anterior-posterior levels of DR. DRD, dorsal part of DR; DRV, ventral part of DR; DRDc, core of DR; DRDsh, shell of DR; DRVL, ventrolateral part of DR; DRC, caudal part of DR; DRI, interfascicular part of DR; a, anterior; p, posterior.

2) Dorsal raphe and regulation of behaviour

A diverse variety of neurons populate different DR subregions, have slightly variable projection targets in the rostral brain, and play distinct roles in the control of behaviour. Because selective serotonin reuptake inhibitors (SSRIs), such as fluoxetine, are the main pharmacological treatments for depression and anxiety disorders, the serotonergic system in the DR has been extensively studied using animal models [213].

The DRDsh has been shown to contain Vglut3^{+} non-serotonergic glutamatergic neurons that innervate the VTA and SNpc dopaminergic neurons and promote reinforcement learning and reward [214, 215]. Additionally, DRI efferent structures include the medial prefrontal cortex, hippocampus, and medial septum and has been implicated in depression [216]. The DRV innervates the olfactory bulb as well as cortical areas including the prefrontal, piriform and primary motor cortex. Optogenetic activation of these serotonergic neurons decreases depression-like behaviour in the forced swim test, reinforcing the idea that it is involved in the regulation of mood [212, 217]. Interestingly, the DRD and DRVL have been implicated to have distinct roles in the regulation of anxiety that is basis of the Deakin/Graeff hypothesis [218]. Craske and colleagues define the features of anxiety disorders as "*excessive and*

enduring fear, anxiety or avoidance of perceived threats, and can also include panic attacks" [219]. Multiple individual disorders that are classified under anxiety disorders include panic disorder, generalized anxiety disorder, specific phobias, and separation anxiety disorder [220]. DRVL serotonergic neurons project to the dorsal periaqueductal grey where they inhibit behaviours associated with panic disorder, including freezing and flight [221]. The DRD, on the other hand, innervates the central amygdala and facilitates conflict anxiety-like behaviour (avoidance) and is implicated in general anxiety disorder. For example, optogenetic activation of serotonergic DRD neurons that project to the amygdala causes increased anxiety-like behaviour in the elevated plus maze, where mice avoid more open arms, suggesting that serotonin has an anxiogenic effect in the amygdala [217]. However, the regulation of anxiety is complex and additional rostral brain structures are involved and receive collateral projections from various parts of the DR, including the lateral septum, prefrontal cortex, bed nucleus of the stria terminalis (BNST) and ventral hippocampus [222, 223]. Interestingly, utilization of several mouse lines, where serotonergic neurons are lost or synthesis of serotonin impaired, has shown that overall serotonin might have an anxiogenic effect, as these mice demonstrate reduced anxiety-like behaviour and explore more open arms in the elevated plus maze [224, 225].

The DR and serotonin concentrations in the brain also influence fear learning during fear conditioning where an aversive stimulus (foot shock) becomes associated with a certain environmental (contextual) or auditory cue. This associated learning involves initial acquisition (conditioning) and consecutive consolidation. Retrieval and extinction of fearful memories are determined by the freezing behaviour of the animal [226-228]. Increased acquisition and reduced extinction of fearful memories has been suggested as one of the causes of post-traumatic stress disorder (PTSD) [229]. Activation of DRD neurons that innervate the amygdala enhances fear learning. This was demonstrated by an increased freezing response in mice subjected to cued fear conditioning [217]. However, depletion of serotonin in the basolateral amygdala results in a reduction in the acquisition and retrieval of fearful memories, as evidenced by reduced freezing behaviour [230]. It has been further shown that a global depletion of brain serotonin causes an increased acquisition and decreased extinction of fearful memories during contextual fear conditioning [224, 225].

3. AIMS OF THE STUDY

GATA2, and GATA3 TFs, along with their co-regulators TAL1 and TAL2, are expressed in embryonic ventral r1 where several distinct cell types are generated that, in turn, give rise to multiple brain structures in the ventral midbrain and anterior hindbrain. However, the role of these factors in the development of r1-derived neurons has remained elusive. We hypothesize, firstly, that similar to the hematopoietic system, the function of GATA TFs is modulated by their cofactors, and secondly, that this results in the diversification of neuronal subtypes dependent them.

The specific aims of this study were:

1. To characterize the heterogeneity of neuronal precursors in ventral r1 at E12.5
2. To study whether GATA2, GATA3 or their co-regulators TAL1, ZFPM1 and ZFPM2 are required for the development of GABAergic neurons in r1
3. To investigate the role of GATA TFs and their co-regulators in the differentiation of serotonergic and glutamatergic neuronal subtypes in the DR.

4. MATERIALS AND METHODS

4.1. Methods

Methods used in this study are listed in Table 1. Detailed descriptions of the methods utilised can be found in the indicated studies.

Table 1. Methods.

Method	Publication
Immunohistochemistry (IHC)	I-IV
in situ hybridization (ISH) on paraffin sections	I-IV
in ovo electroporation	I
BrdU and EdU labelling	II, III
Double ISH	II-IV
PCR genotyping	I-IV
Microscopy and quantification	I-IV
Cell dissociation for single-cell RNA sequencing	III
Statistical analysis	I-IV

4.2. Materials

Mouse strains, primary antibodies and *in situ* probes used in this study are listed in Tables 2-4.

Table 2. Mouse lines used in this study.

Mouse line	Description	Reference	Publication
En1Cre	Cre recombinase under the control of *En1* promoter	[231]	I-IV
Nkx2-2Cre	Cre recombinase under the control of *Nkx2-2* promoter	[232]	II
Gbx2CreERT2	tamoxifen-inducible Cre recombinase under the control of *Gbx2* promoter	[233]	I
Tal1flox	*Tal1* allele flanked by loxP sites to allow conditional inactivation of *Tal1*	[234]	I, III
Gata2flox	*Gata2* allele flanked by loxP sites to allow conditional inactivation of *Gata2*	[235]	I-II

Gata3flox	*Gata3* allele flanked by loxP sites to allow conditional inactivation of *Gata3*	[236]	I-II
Zfpm1flox	*Zfpm1* allele flanked by loxP sites to allow conditional inactivation of *Zfpm1*	[74]	IV
Zfpm2flox	*Zfpm2* allele flanked by loxP sites to allow conditional inactivation of *Zfpm2*	[237]	III, IV
R26RTdTomato	*ROSA26Sor* locus containing a loxP-flanked STOP cassette upstream of the *RFP* gene	[238]	II, III

Table 3. Primary antibodies used in this study.

Antibody	**Host**	**Source**	**Cat. nr.**	**Publication**
5-HT	rabbit	Immunostar	20080	I, II, IV
BrdU	mouse	GE Healthcare	RPN20AB	III
Calbindin	mouse	Swant	CB300	III
Cart	rabbit	Phoenix Pharmaceuticals	H-003-60	II
ChAT	goat	Millipore	AB144P	I
Ctip2	rat	Abcam	ab18465	III
Foxo1	rabbit	Cell Signaling Technology	2880	III
FoxP1	mouse	Abcam	ab32010	III
FoxP1	rabbit	Abcam	ab16645	I, III
Gata2	rabbit	Santa Cruz Biotechnology	sc-9008	III, IV
Gata3	mouse	Santa Cruz Biotechnology	sc-268	III, IV
GFP	rabbit	Abcam	ab290	I
HuC/D	mouse	Molecular Probes	A21271	III
Isl1	mouse	DSHB	40.4D6	II
Nkx2-2	mouse	DSHB	74.5A5	I-III
Nkx6-1	rabbit	DSHB	F55A10	I-III
Olig2	goat	Neuromics	GT15132	I, III
Parvalbumin	goat	Swant	PVG213	III
Pax3	mouse	DSHB	AB_528426	III
Pax7	mouse	DSHB	AB_528428	III
pHistone H3	rabbit	Millipore	06-570	III
RFP	rabbit	Rockland	600-401-379	I-III
Sox2	mouse	Abcam	ab79351	III

Sox2	rabbit	Millipore	AB5603	III
TH	mouse	Millipore	MAB318	I, III, IV
TH	rabbit	Chemicon	AB152	I, III, IV
Vsx2	sheep	Abcam	ab16141	I, III
Zfpm2	rabbit	Santa Cruz Biotechnology	sc-10755	III, IV
Zfpm2	mouse	Santa Cruz Biotechnology	sc-398011	III

Table 4. *In situ* probes used in this study.

Probe	**Reference/Source**	**Publication**
chicken *Gad1*	[8]	I
chicken *Tal1*	a gift from David Rowitch	I
mouse *Gad1*	[239]	II, III
mouse *Pdzrn4*	Source BioScience (RIKEN ID6820442M07)	III
mouse *Pet1*	clone UI-M-BH3-avj-b-02-0-UI.s1	I, II
mouse *Sert*	Allen Brain Atlas (RP_071204_04_G10)	I, II, IV
mouse *Sox14*	Source BioScience (IMAGp998A2414391Q)	I, III
mouse *Tal1*	Source BioScience (IRAVp968D09118D)	III
mouse *Tph2*	[140]	II
mouse *Vglut2*	[239]	I-III
mouse *Vglut3*	Guimera, unpublished	II, IV
mouse *Zfpm1*	Source BioScience (IMAGE ID3585094)	III, IV
mouse *Zfpm2*	Source BioScience (IRAVp968B06115D)	I, III

5. RESULTS AND DISCUSSION

5.1. Analysis of progenitor domains, heterogeneity of ventral r1, and characterization of nuclei derived from ventral r1 (I-III)

5.1.1. Identification of progenitor domains in ventral r1 (II)

In early embryonic development, the ventral r1 contains multiple domains that give rise to different cell types and nuclei in the adult brain. Previously it has been determined that the most ventral r1 region produces GABAergic and glutamatergic neurons, as well as serotonergic neurons [119, 122-124]. To improve our understanding of early ventral r1 patterning and characterize its progenitor domains, we analysed gene expression in the ventricular zone of mouse ventral r1 and utilised genetic fate mapping to characterize cell types produced from these progenitors. The patterning of the spinal cord is well characterized and shares many similarities to ventral r1 (Figure 7 and Figure 10) [240]. The ventricular zone of the ventral spinal cord expresses several homeodomain TFs including *Nkx2-2*, *Nkx2-9*, *Nkx6-1* and *Nkx6-2* that are involved in neuronal specification [241-243]. We analysed the expression of these factors in mouse ventral r1 between E10.5-E12.5. Based on this we were able to divide the ventricular zone into three distinct progenitor domains. Located from the ventral to dorsal r1, these were defined as the rp3 (rhombencephalic progenitor domain 3), the rpvMN (rhombencephalic visceral motor neuron progenitor domain), and the rp2 (rhombencephalic progenitor domain 2). *Nkx2-2* is expressed in the rp3 and rpvMN domains, *Nkx2-9* is specific to the rpvMN domain, whereas *Nkx6-1* is expressed in the dorsal part of the rp3, in the rpvMN, and also in the rv2 domain (II/Fig.1).

Previously, it has been shown that in more caudal rhombomeres (r2-r8, except r4) Nkx2-2^{+} progenitors generate first *Isl1* positive visceral motor neurons and then proceed to produce serotonergic neurons, while in the r1, motor neuron generation does not precede that of serotonergic neurons and therefore only serotonergic neurons are produced [198, 203, 241]. Contrary to the common belief that no motor neurons are derived from r1, we identified *Isl1* positive cells in close proximity to the rp3 and the rpvMN domain at stages E10.5-E11.5 (II/Fig.1). Also, it has been shown how in more caudal rhombomeres (r2-r3), the dorsally located Nkx2-2^{+};Nkx2-9^{+} progenitor domain continues to generate motor neurons, while the Nkx2-2^{+};Nkx2.9^{-} domain starts to produce serotonergic neurons [198] and the Nkx2-2^{+} domain starts to also produce oligodendrocytes at E12.5-E13.5 [244]. Instead of motor neuron markers, we found oligodendrocyte specific makers *Olig2, Sox10 (SRY (sex determining region Y)-box 10)* and *Pdgfra* (*Platelet derived growth factor receptor, alpha polypeptide*) in the Nkx2-2^{+};Nkx2-9^{+} (rpvMN) progenitor domain at E12.5 (II/Fig.1). By using the *Nkx2-2$^{Cre/+}$;R26R^{TdTomato}* mouse line, we identified cell types derived from Nkx2-2^{+} progenitors and found that Isl1^{+} motor neurons, Pet1^{+} serotonergic neurons and Olig2^{+} oligodendrocytes are all derived from Nkx2-2^{+} progenitors (II/Fig.1). Interestingly, Isl1^{+} cells in the trochlear nucleus at E18.5 were also labelled, indicating that they are

produced from Nkx2-2^{+} cells, possibly from r1. Accordingly, the trochlear motor neurons have been shown to arise from the anterior r1 in chicken [202] and more recently in mouse [93]. These results show that ventral r1 can be divided into different progenitor domains and produces several distinct cell types located in separate post-mitotic domains (mantle zone areas). At E10.5, Nkx2-2^{+} progenitors produce Isl1^{+} motor neurons. At E12.5, Nkx2-2^{+};Nkx2-9^{+} progenitors generate oligodendrocytes (rvMN domain), Nkx2-2^{+};Nkx2-9^{-} progenitors give rise to serotonergic neurons (rV3 domain) and the Nkx6-1^{+} rp2 domain produces both GABAergic and glutamatergic neurons (rV2 domain) (Figure 10).

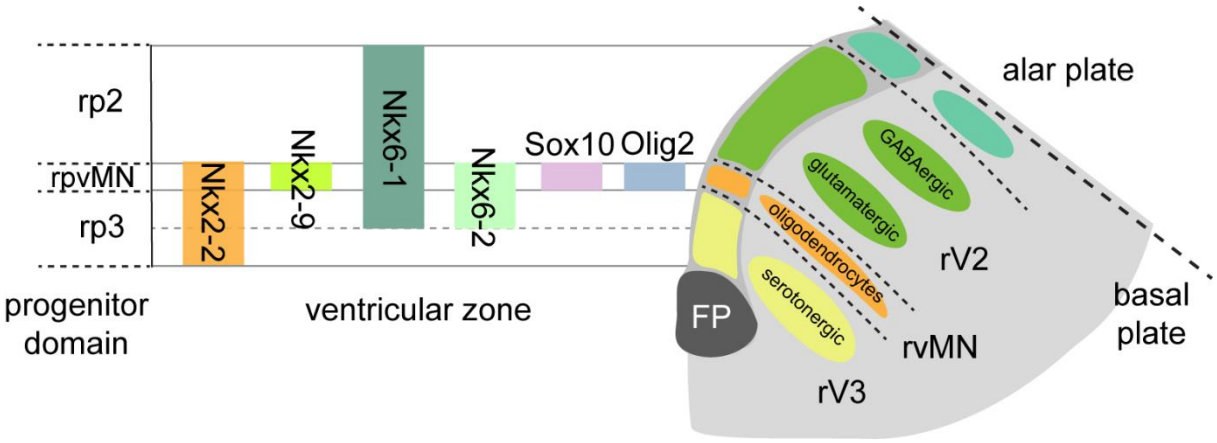

Figure 10. Progenitor domains in the ventral r1 at E12.5. The basal plate of r1 contains at least four distinct progenitor domains (the most dorsal progenitor domain will not be characterized in this study) that express different TFs and give rise to various cell types. The most ventral rp3 domain generates serotonergic neurons located in the post-mitotic mantle zone area rV3. The rpvMN progenitor domain gives rise to oligodendrocytes in the rvMN and dorsally located progenitors in rV2 domain produce both GABAergic and glutamatergic neurons that are intermingled in the mantel zone area (rV2). FP, floor plate; rp3, rhombencephalic progenitor domain 3; rpvMN, rhombencephalic visceral motor neuron progenitor domain; rp2, rhombencephalic progenitor domain 2; rV3, rhombencephalic V3 domain; rvMN, rhombencephalic vMN domain; rV2, rhombencephalic V2 domain.

5.1.2. Heterogeneity of ventral r1 at E12.5: ventral r1 contains at least 21 cell populations (III)

To characterize different cell populations in the developing embryonic r1, we conducted single-cell RNA sequencing (scRNAseq) on mouse ventral r1 tissue at E12.5. In this method, r1 is separated from the rest of the primordial brain and broken down into a solution containing single cells. Each cell is then encapsulated in a distinct environment (droplet) allowing us to determine individual gene expression profiles by mRNA sequencing analysis [245]. At E12.5, cells produced in r1 have not yet started migrating towards the midbrain. Furthermore, at this stage the midbrain-hindbrain boundary constriction still physically confines them and allows for more precise isolation [246]. To better define the role of TAL1 in r1, we conducted scRNAseq on both *Tal1* conditional mouse mutants *Tal1*^{CKO}

(*En1*$^{Cre/+}$;*Tal1*$^{flox/flox}$) and *Control* (*En1*$^{Cre/+}$;*Tal1*$^{flox/+}$) ventral r1 tissue to characterize its heterogeneity at E12.5 and identify cells dependent on its expression. In *Tal1*^{CKO} tissue, *Tal1* is inactivated in *En1* (Engrailed 1) positive tissue such as midbrain and r1 [231].

By analysing individual cell mRNA sequences and comparing them to others, we were able to identify 21 distinct clusters (cell populations) with similar expression patterns (III/Fig.1). Co-expressed genes were used to define each population. Three of the identified clusters expressed progenitor specific markers including *Nestin*, *Ascl1*, *Nkx6-1* (genes known to be expressed in the r1ventricular zone) [124, 131], genes important in cell-cycle regulation such as *Cenpa* (*Centromere protein A*), *Ccnb1* (*Cyclin B1*) [247-249], and the Notch pathway genes *Notch1*, *Dll3* and *Hes5* that contribute to the preservation of neuronal stem cells [250, 251]. This indicated that these clusters are progenitors, while the other 18 clusters expressed the marker *Tubb3* (*Tubulin beta 3*) and were considered post-mitotic (III/Fig.1) [252].

The post-mitotic clusters, in addition to *Tubb3*, expressed glutamatergic (*Vglut2*$^{+}$), GABAergic (*Gad1*$^{+}$) or serotonergic (*Vglut3*$^{+}$, *Pet1*$^{+}$, *Gata2*$^{+}$, *Gata3*$^{+}$, *Zfpm1*$^{+}$) markers (Figure 11). Independent validation of glutamatergic and GABAergic clusters by ISH demonstrated that they have multiple developmental origins in r1 (several domains in r1 generate GABAergic and glutamatergic neurons, while serotonergic neurons have only one origin in the r1 (rp3 progenitor domain)). Seven glutamatergic clusters were positioned more laterally and dorsally in the r1 and could be further divided into *Lmx1b*$^{+}$ (two clusters) and *Lhx9*$^{+}$ (five clusters) populations (III/Fig.2). Previous studies have demonstrated that *Lhx9* expressing glutamatergic neurons are derived from the *Atoh1* positive rhombic lip region of dorsal r1, migrate tangentially to ventral r1, and generate multiple nuclei in the pons that are components of the auditory pathway (lateral lemniscus and superior olive nucleus), or regulate arousal/wakefulness (parabrachial nucleus and PPTg) [253-256].

Similarly, we identified GABAergic clusters whose developmental origin was outside of the rp2 domain, presumably in dorsal r1. These were *Skor1* (*SKI family transcriptional corepressor 1*) and *Zic1* (*Zinc finger protein of the cerebellum 1*) double positive (two clusters) and *Otp* (3 clusters) positive populations (III/Fig.2). Although we also found some *Skor1* positive cells in the rV2 domain, they were not GABAergic. GABAergic *Skor1*$^{+}$ cells were confined to the more dorsal region of r1 and were likely derived from adjacent *Pax3*$^{+}$;*Zic1*$^{+}$ progenitors in the ventricular zone. The three *Otp*$^{+}$ clusters expressed differentially *Cntn2* (*Contactin 2*), *Foxo1* (*Forkhead box O1*) and *Ntn1* (*Netrin 1*). These cell groups seemed to form a migratory stream of cells from dorsal r1 to ventral r1. We found that *Cntn2*$^{+}$ cells were positioned dorso-laterally, *Foxo1*$^{+}$ cells had a higher presence in the rV2 domain, and *Ntn1*$^{+}$ cells were positioned ventromedially, near the serotonergic rV3 domain. All *Otp*$^{+}$ clusters also expressed *Pax7* at lower levels and earlier studies have shown that *Pax7* and *Otp* positive cells in the ventral r1 are derived from the dorsal Pax7^{+} ventricular zone, migrate ventrally and give rise to the IPN in the chicken [122]. Previously, a *Pitx2* (*Paired-like homeodomain transcription factor 2*) positive GABAergic cell population was characterized in ventral r1 [124]. Additional analysis revealed that cells identified as

Ntn1^{+} also expressed *Pitx2*, indicating that it might be the same population previously described (III/Fig.S1).

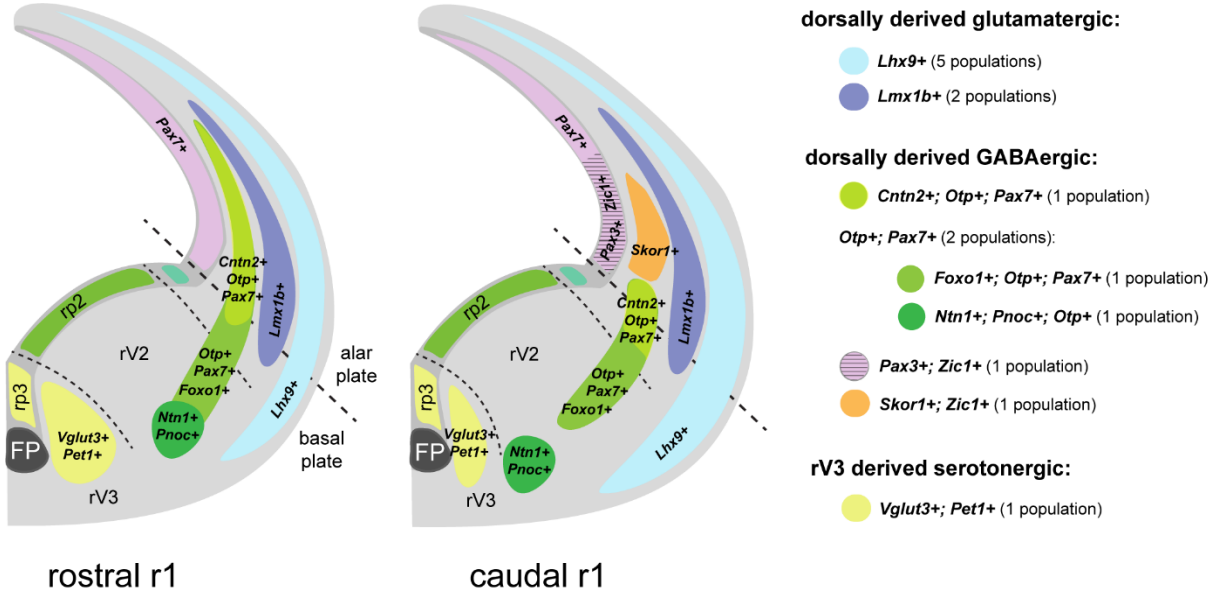

Figure 11. Schematic overview of cell populations flanking the rV2 domain in the mouse rostral and caudal r1 at E12.5 detected with scRNA-seq and validated with ISH or IHC. The main specific makers of each population is indicated. Cell populations derived from rV2 (5 populations) or identified progenitor cell populations (3 populations) are not shown.

5.1.3. Characterization of rV2-derived cell populations (III)

The remaining five clusters identified were positioned in the rV2 domain and presumably derived from the rV2 domain (Figure 12). Previously, the rV2 domain in r1 was shown to contain neuronal precursors that express *Gata2* and *Gata3* along with their co-regulators *Tal1* and *Tal2* [10, 123]. Two of the rV2 clusters expressed the glutamatergic markers *Vglut2*, *Nkx6-1* and *Vsx2*. One of these also expressed the markers *Skor1*, *Lhx4*, *Shox2* (*Short stature homeobox 2*) and *Sox14*, while the other cluster expressed *Pax5*, *Asic4* (*Acid-sensing (proton-gated) ion channel family member 4*) and *Pou6f2* (*POU domain, class 6, transcription factor 2*). We found these markers to be expressed broadly in the rV2 domain. Three other clusters expressed *Tal1* (III/Fig.1, 3). Of these, two expressed *Gad1*, indicating a GABAergic identity. One of these, also expressed the TFs *Gata3*, *Gata2*, *Zfpm1*, *Zfpm2*, *Sox14*, *Asic4*, *Otx1* and *Pax5*, showing that like GATAs and TALs, GATA cofactors *Zfpm1* and *Zfpm2* are also expressed at E12.5 in GABAergic cells in the rV2 domain of ventral r1. Similar to the glutamatergic clusters, we found these to be expressed widely over the rV2 domain. The second GABAergic cluster expressed *Sall3* (*Spalt like transcription factor 3*) and *Gata3*. Interestingly, we found

Sall3 to be expressed only in the medial region of the rV2 domain, in close proximity to the ventricular zone. This demonstrates that the rV2 domain can be divided dorso-ventrally into *Sall3*$^{+}$ and *Sall3*$^{-}$ regions (III/Fig.5).

The third *Tal1* positive cluster did not express *Gad1* nor *Vglut2*, but did express genes *St18* (*Suppression of tumorigenicity 18*), *Nkx6-1* and Notch pathway or corresponding target genes such as *Hes5*, *Dll3*, *Pdzk1ip1* (*PDZK1 interacting protein 1*, also known as *Map17*), *Notch1* and *Mfng* (*MFNG O-fucosylpeptide 3-beta-N-acetylglucosaminyltransferase*) [257]. This expression pattern was similar to that seen in clusters containing progenitors but expressed additionally the post-mitotic marker *Tubb3*. Based on this and the pseudotemporal ordering of cells, this cluster was presumed to contain post-mitotic immature neurons (III/Fig.3,4). It has been shown that Notch signalling determines the cell fate specification of excitatory or inhibitory interneurons in post-mitotic cells of the spinal cord. Adjacent immature neurons in the developing spinal cord interact through the Notch pathway components NOTCH1 (Notch receptor) and DLL4 (Notch ligand) on the cell surface. In response to this interaction some cells accumulate higher *Notch1* expression, start to express *Tal*, and become Gata2^{+}Gata3^{+}Tal1^{+} GABAergic neurons. In contrast, cells that express higher levels of DLL4 develop into Vsx2^{+} glutamatergic neurons [153]. Similar cell fate determination through Notch signalling may also occur in r1, when immature precursors must decide between a GABAergic and glutamatergic fate. However, this hypothesis needs to be confirmed by additional studies.

To summarize, scRNAseq analysis of ventral r1 *Control* tissue allowed us to characterize neuronal populations, their specific markers as well as determine the exact expression patterns of the GATA factors *Gata2*, *Gata3* and their regulators *Tal1*, *Zfpm1*, *Zfpm2* in cell groups that could help us further investigate their role in r1 neuron development.

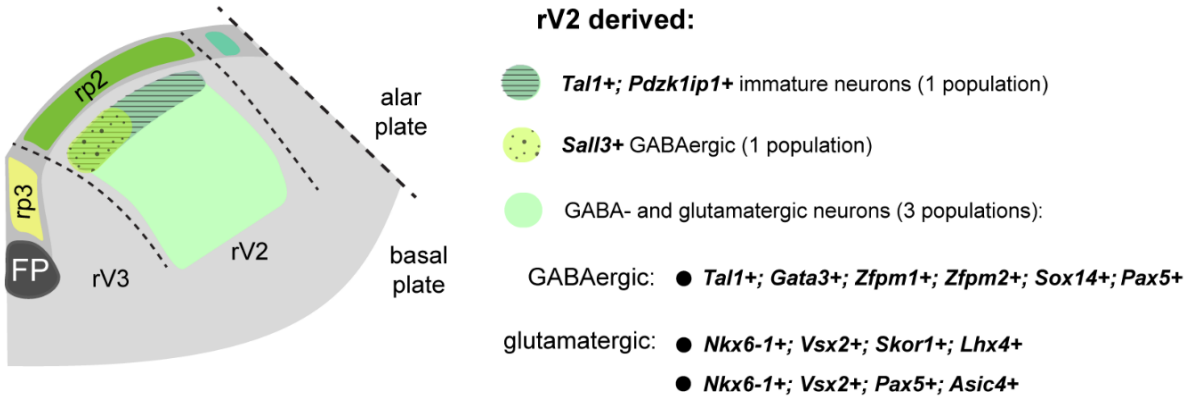

Figure 12. Schematic overview of five cell populations in the rV2 domain of r1 at E12.5 identified with scRNA-seq and validated with ISH or IHC. The specific genes expressed by each populations are shown. Tal1^{+}Pdzk1ip1^{+} populations of cells are located near the ventricular zone but also intermingled with cells belonging to other populations that are similarly positioned near ventricular zone. Sall3^{+} GABAergic cells are aggregated in the

medial part of the rV2 domain near the ventricular zone. The rest of the cell populations are intermingled throughout the rV2 domain.

5.1.4. Ventral r1 gives rise to multiple nuclei in ventral midbrain and rostral hindbrain (I, III)

Having shown that r1 contains multiple distinct glutamatergic and GABAergic cell populations at E12.5 and identified specific markers for these, we decided to determine which nuclei they give rise to in the more mature brain by analysing the midbrain and the anterior hindbrain at later embryonic stages. It had been previously demonstrated that pSNpr GABAergic neurons are derived from r1 and are *Tal1*-dependent as these neurons are absent in $Tal1^{CKO}$ embryos at E18.5, while these neurons develop normally in *Gata2* and *Gata3* single mutants [123] (also I/Fig.3,4). However, it has been suggested that the aSNpr originates from the diencephalon or the midbrain and its development is *Tal1*-independent and requires *Gata2* [123, 172]. To investigate this, we characterized gene expression in the aSNpr and the pSNpr at E18.5. At this stage, the aSNpr expresses *Six3*, *Tal1*, FOXP1 (Forkhead box P1), GATA3 and *Zfpm1*, while the pSNpr expresses *Tal1*, *Zfpm2*, *Sox14* (laterally), *Pax5*, *BCL11B*, *En1* and *Gata3* (I/Fig.3,4 and III/Fig.S6). Utilization of the inducible $Gbx2^{CreERT2/+}$;$R26R^{TdTomato}$ mouse line allowed us to identify r1-derived neurons, as *Gbx2* is expressed specifically in the hindbrain including r1 [246]. Analysis of these showed that pSNpr neurons originate from r1, whereas aSNpr neurons are derived from other areas, as previously proposed (I/Fig.3). *Tal1*-dependent development of pSNpr neurons indicates they may be derived from GABAergic cells in the rV2 domain of r1 (the only region where *Tal1* is expressed in r1). Using scRNAseq, we identified one GABAergic population that expressed *Tal1* as well as other pSNpr specific markers such as *Zfpm2*, *Sox14* and *Pax5*, indicating that they could indeed be derived from this population.

We also characterized the expression pattern of three other GABAergic nuclei, specifically the RMTg, the VTg and the DTg. At E18.5, we identified FOXP1, *Sox14* and SOX2 as novel markers for RMTg neurons and showed that, as was the case with the pSNpr, the RMTg also originates from r1 (I/Fig.5,6,S3). Similarly, the novel specific markers of VTg are ZFPM2 and *Tal1*, while DTg expresses FOXO1 and PAX3 (III/Fig.6). Analysis of the developmental origin of these two nuclei revealed that VTg neurons are derived from the rV2 domain, while DTg cells are generated from $Pax7^{+}$ progenitors in the dorsal r1 (possibly the identified $Foxo1^{+}Pax7^{+}Otp^{+}$ population in the scRNAseq study) and migrate ventrally between the ventricular zone of the rV2 domain and VTg cells (III/Fig.6,S5).

At E12.5, we were able to identify two glutamatergic populations within the rV2 domain. Both expressed *Nkx6-1* and *Vsx2*. To find out what these give rise to, we tracked the expression of NKX6-1 and VSX2 from E12.5 to E18.5. At these stages NKX6-1 and VSX2 cells migrated from the rV2 domain and at later stages expressed the glutamatergic marker *Vglut2* and populated the IPN and the LDTg (I/Fig.8,S6). The IPN contains both glutamatergic and GABAergic ($Pax7^{+}$) neurons. Interestingly, it has been previously shown in the chicken how these cells have different developmental origins: $Nkx6\text{-}1^{+}$

cells arise from the rV2 domain, while Pax7^{+} GABAergic cells are derived from dorsal r1 [122]. As NKX6-1 and VSX2 marked both glutamatergic populations (as identified by scRNAseq), we were unable to distinguish the exact lineage of IPN and LDTg neurons. Specific genes for these populations at E12.5 (*Skor1*, *Lhx4*, *Shox2*, *Sox14* versus *Pax5*, *Puo6f2*, *Asic4*) were found to be mostly down-regulated at later stages, although *Lhx4* was expressed in LDTg cells at E18.5 (III/Fig.S3).

After describing the different populations of r1 at E12.5, we were able to show the lineage of some of these populations to shed light on what nuclei they will produce at later stages of development. However, temporal changes in gene expression during development are common and additional lineage tracing methods should be employed to confirm and further refine these findings.

5.2. GATA TFs and their co-regulators TAL1 and ZFPM2 regulate the development of GABAergic neurons in r1 (I, III)

5.2.1. TAL1 is a neuron-type selector gene in the rV2 domain (I, III)

Having characterized different cell populations in r1 and analysed what they give rise to at later stages, we chose to investigate whether *Tal1* is also required for or regulates their development. To do this we examined *Tal1*^{CKO} conditional mouse mutants at E12.5 and E18.5. First, we looked at the main markers of the two GABAergic populations that were previously identified using scRNAseq (*Gata3*, *Zfpm1*, *Zfpm2*, *FoxP1* and *Sall3*) in r1 at E12.5. Their expression was significantly reduced in *Tal1*^{CKO} embryos, indicating that in these mutants both types of GABAergic cells were unable to differentiate correctly (I/Fig.1 and III/Fig.5). Interestingly, when we analysed the glutamatergic markers *Vsx2*, *Nkx6-1*, *Skor1* and *Lhx4*, we found that the number of cells expressing these to be increased in r1 at E12.5 (I/Fig.9 and III/Fig.3), indicating an aberrant GABAergic to glutamatergic neuron ratio and a probable deviation to a glutamatergic-like state. To assess whether *Tal1* is able to induce GABAergic development, we used *in ovo* electroporation to ectopically express it specifically in the rV3 domain of r1 that typically produces only serotonergic neurons. We found that following this intervention, this region began to generate GABAergic neurons (I/Fig.2).

We also analysed the development of r1-derived GABAergic and glutamatergic nuclei in *Tal1*^{CKO} mutants at the later stage of development at E18.5. As previously shown, pSNpr GABAergic cells were absent from these animals (I/Fig.4) [123]. Similarly, other GABAergic nuclei such as the RMTg and the VTg, were missing in these mutants (I/Fig.7 and III/Fig.6), while the GABAergic nucleus DTg developed normally, confirming that this nucleus has a *Tal1*-independent developmental origin (III/Fig.6). However, while rV2-derived GABAergic nuclei were missing in the *Tal1*^{CKO} animals, the number of Nkx6-1^{+} and Vsx2^{+} glutamatergic neurons in the IPN and the LDTg increased (I/Fig.10), showing that extra glutamatergic neurons are produced in the rV2 domain in absence of *Tal1*, are maintained at later stages, and are able to migrate to the correct location.

These results suggest that TAL1 might function as a neuron-type selector gene in r1, where it dictates a GABAergic over glutamatergic fate. Similarly, TAL1 has been shown to have the same function during the development of GABAergic neurons in the midbrain (confined to a narrow domain) and the spinal cord [10, 146, 153]. Additionally, the TAL1 regulatory partner GATA2 and the second TAL protein, TAL2, have also been demonstrated to operate as neuron-type selectors during the development of diencephalon and midbrain GABAergic neurons [9, 10, 132]. It is noteworthy that, although *Gata2* and *Tal2* are also co-expressed in r1, they alone do not act as a neuron-type selector genes in r1 as single mutants demonstrate unimpaired development of r1-derived ventral midbrain GABAergic neurons (I/Fig.S2,S5,S7) [10].

5.2.2. GATA2 and GATA3 work redundantly to determine the GABAergic fate in the rV2 domain (I, III)

Unlike TAL1, GATA2 and GATA3 alone are not required for the development of rV2 GABAergic neurons [123] (also I/Fig.S2). However, we hypothesized that GATA2 and GATA3 could work together redundantly, so that when one factor is lost, the other would compensate for its absence. Therefore, we examined GABAergic development in the *Gata2* and *Gata3* conditional double KO mouse line ($En1^{Cre/+};Gata2^{flox/flox};Gata3^{flox/flox}$) at E12.5 and E18.5. As seen in the $Tal1^{CKO}$, $Gata2;Gata3^{DKO}$ animals exhibited a reduction in the number of GABAergic neurons and a downregulation of GABAergic markers (including *Tal1*, *Zfpm1*, *Zfpm2*, FOXP1 and SOX2) was observed in the rV2 domain. In contrast, the number of glutamatergic neurons increased (NKX6-1 and VSX2) (I/Fig.1,9). In addition, pSNpr and RMTg GABAergic neurons were absent, while more Nkx6-1^{+} and $Vsx2^{+}$ cells were present in the IPN and the LDTg of $Gata2;Gata3^{DKO}$ mutants at E18.5 (I/Fig.7,10). This shows that simultaneous inactivation of *Gata2* and *Gata3* causes a phenotype comparable to that seen in *Tal1* mutant animals and demonstrates that they act redundantly to determine a GABAergic over glutamatergic cell fate in the rV2 domain of r1.

5.2.3. ZFPM2 is required for the development of GABAergic nuclei from the rV2 domain (III, IV)

Knowing that GATA cofactors *Zfpm1* and *Zfpm2* are also expressed by GABAergic neurons located in the rV2 domain of r1 at E12.5 (I/Fig.1 and III/Fig.3), we decided to investigate how ZFPM1 and ZFPM2 affect the development of rV2 GABAergic neurons using *Zfpm1* and *Zfpm2* conditional single and double mutants $Zfpm1^{CKO}$ ($En1^{Cre/+};Zfpm1^{flox/flox}$), $Zfpm2^{CKO}$ ($En1^{Cre/+}Zfpm2^{flox/flox}$) and $Zfpm1;Zfpm2^{DKO}$ ($En1^{Cre/+};Zfpm1^{flox/flox};Zfpm2^{flox/flox}$). Contrary to what was seen in $Tal1^{CKO}$ and $Gata2;Gata3^{DKO}$ animals, we did not observe any changes in number of GABAergic neurons nor in the expression of GABAergic and glutamatergic markers in r1 at E12.5 (data not shown). This demonstrates that in the absence of the *Zfpm1* or *Zfpm2*, GABAergic neurons were still able to differentiate and that

ZFPM1 and ZFPM2 are not involved in the determination of a GABAergic vs. glutamatergic cell fate as was observed with TAL1 and GATA2/GATA3.

However, analysis of these mutants at E18.5, revealed that the development of the pSNpr, the VTg and the RMTg was impaired in *Zfpm2*^{CKO} animals. pSNpr and VTg GABAergic neurons were absent, whereas the RMTg contained fewer FoxP1^{+} and Sox14^{+}Sox2^{+} positive neurons in *Zfpm2*^{CKO} mutants at E18.5 (III/Fig.7). A comparison of results from E12.5 and E18.5 old *Zfpm2*^{CKO} animals suggests that pSNpr, VTg and RMTg GABAergic cells are produced normally at early stages (E12.5), but die at later stages (before E18.5). However, we were unable to detect any apoptotic markers at E14.5 (data not shown). Further analysis of *Zfpm2*^{CKO} embryos at E14.5 revealed that Sox14^{+}Sox2^{+} positive RMTg neurons exhibited a more scattered distribution and did not from a compact nucleus (data not shown). Furthermore, Pax5^{+} pSNpr neurons were located correctly (near the aSNpr) at E14.5. This indicates the possibility that pSNpr-like GABAergic neurons are able to differentiate from the rV2 domain but do not form a proper pSNpr at later stages, perhaps due to a failure to migrate to their appropriate positions. However, we did not find any misplaced cell groups whose gene expression pattern matched the usual pSNpr markers in the midbrain or the hindbrain (data not shown).

We did not detect any defects in GABAergic neuron development in *Zfpm1*^{CKO} animals. In *Zfpm1*^{CKO} embryos, rV2-derived GABAergic and glutamatergic nuclei were able to form normally, showing that ZFPM1 is not required for this function (IV/Fig.2). However, *Zfpm1;Zfpm2*^{DKO} embryos had identical phenotypes to those of *Zfpm2*^{CKO} embryos, likely caused entirely by the absence of *Zfpm2* (IV/Fig.2).

It is evident from these results that ZFPM1 and ZFPM2 do not act as a neuron-type selectors, even redundantly, in the rV2 domain of r1, on account that we able to detect regular numbers of GABAergic and glutamatergic neurons at E12.5 in embryos that lack them. However, ZFPM2 does appear to be involved in the generation of RMTg GABAergic neurons and has previously been shown to play a role in the regulation of corticothalamic projection neuron (CThPN) differentiation in the mouse neocortex [148]. ZFPM2 seems to be required for either the survival or the positioning of pSNpr and VTg GABAergic cells.

5.3. GATA2, GATA3 and ZFPM1 control different aspects of serotonergic neuron development in r1 (II, IV)

5.3.1. Serotonergic rV3 domain in r1 contains distinct Vglut3 and Sert positive serotonergic populations (II)

The adult DR is a diverse nucleus, containing neurons that differ in gene expression, morphology, electrophysiological characteristics, projections and neurotransmitters [211, 212, 217, 258]. Analyses of the expression pattern of two serotonergic markers, *Vglut3* and *Tph2*, have shown that

the adult mouse DR contains Vglut3$^-$Tph2$^+$ and Vglut3$^+$Tph2$^+$ serotonergic neurons and that Vglut3$^+$ non-serotonergic glutamatergic neurons project to VTA and regulate the reward pathway [214, 215, 259]. We sought to determine when this heterogeneity is achieved and so examined the expression of *Vglut3* together with the serotonergic markers *Sert*, *Tph2* and *Pet1* in neurons originating from the rV3 domain of r1 during DR development [119]. We found that as soon as E11.5-E12.5, serotonergic precursors could be separated into two distinct populations in the rV3 domain. While those located dorsally expressed *Vglut3*, those in the ventral region were observed to express *Sert* and *Tph2* (Figure 13). Both populations were also *Pet1* positive (II/Fig.2). This demonstrates that serotonergic neurons are heterogeneous early in development. In addition, we discovered that the DR is composed of Sert$^+$ (serotonergic) and Vglut3$^+$ (non-serotonergic glutamatergic neurons) single positive cells and that Sert$^+$Vglut3$^+$ (serotonergic) double positive cells are present at E18.5 (II/Fig.8).

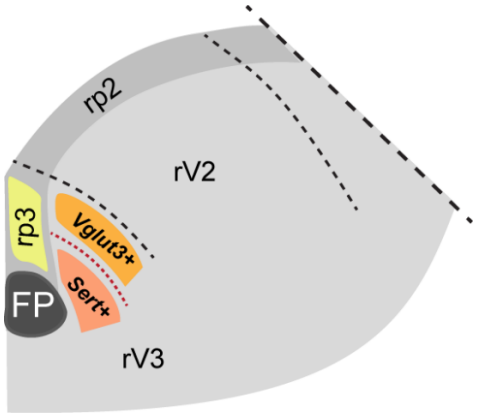

Figure 13. At E12.5, serotonergic neurons in the rV3 domain of r1 can be divided into two populations. Dorsally located serotonergic neurons express *Vglut3*, while ventrally positioned serotonergic neurons express *Sert* and *Tph2*. The domains rpvMN and rvMN are not shown. FP, floor plate; rp3, rhombencephalic progenitor domain 3; rp2, rhombencephalic progenitor domain 2; rV3, rhombencephalic V3 domain; rV2, rhombencephalic V2 domain.

Furthermore, Vglut3$^+$ and Sert$^+$ serotonergic precursor populations were found to divide the rV3 domain into ventral and dorsal regions at E12.5, indicating that they may be derived dorso-ventrally from different rp3 progenitors. Interestingly, we observed that *Nkx6-1* and *Nkx6-2* are only expressed in more dorsal part of rp3 progenitors at E12.5 where they divide the area into Nkx6-1$^+$Nkx6-2$^+$ (dorsal) and Nkx6-1$^-$Nkx6-2$^-$ (ventral) populations (Figure 10). We analysed expression of *Nkx6-1* together with *Vglut3* and *Sert* at E12.5. *Vglut3* positive serotonergic neurons were found located near Nkx6-1$^+$ progenitors, while the lower *Sert* positive neurons were close to Nkx6-1$^-$ progenitors (II/Fig.3). This suggested that the Nkx2-2$^+$ progenitor domain could be heterogeneous along its dorsal-ventral axis and that Vglut3$^+$ and Sert$^+$ serotonergic populations may be derived from different rp3 progenitors. However, Nkx6-2 lineage-tracing should be used to confirm this hypothesis (at earlier stages Nkx6-1 was expressed also in the ventrally located rp3 progenitors, while Nkx6-2 was not).

5.3.2. GATA2 acts as neuron-type selector of the serotonergic neurons in r1 (II)

Two distinct populations of serotonergic precursors are present in r1 at E12.5 with this heterogeneity being maintained and enhanced at later stages of development so that the mature DR is composed of different serotonergic and non-serotonergic glutamatergic neurons (II/Fig.2) [212, 214]. Previously, GATA TFs have been shown to regulate the development of serotonergic neurons in the hindbrain. GATA2 is required to specify serotonergic identity, while GATA3 has been shown to be important only for the differentiation of caudal serotonergic neurons [120, 147]. Therefore, we wanted to verify whether GATA2 and GATA3 are also important for the acquisition of serotonergic neuron heterogeneity. First, we analysed the expression of *Gata2* and *Gata3* in two serotonergic populations (Vglut3$^+$ and Sert$^+$) that we had identified in the rV3 domain at E12.5. At this stage, *Gata2* and *Gata3* are both expressed in post-mitotic serotonergic precursors in the rV3 domain. However, GATA2 levels were higher in the Vglut3$^+$ population but weak in the Sert$^+$ population, while GATA3 was observed at comparable levels in both populations at E12.5 (II/Fig.4). At E16.5, both *Gata2* and *Gata3* were found to be expressed in Vglut3$^+$ and Sert$^+$ cells (II/Fig.4).

Next, we analysed serotonergic development using *Gata2* and *Gata3* conditional single and double KO mouse lines *Gata2*^{CKO} (*En1*$^{Cre/+}$*;Gata2*$^{flox/flox}$), *Gata3*^{CKO} (*En1*$^{Cre/+}$*;Gata3*$^{flox/flox}$) and *Gata2;Gata3*^{DKO}. Inactivation of *Gata2* at E12.5 resulted in the loss of all serotonergic neurons from r1 (both Vglut3$^+$ and Sert$^+$ populations) (II/Fig. 5), consistent with previous studies [9, 120]. Additionally, cDNA microarray and *in situ* hybridization (ISH) analyses confirmed that the main serotonergic markers *Vglut3*, *Sert*, *Pet1* and *Tph2* were down-regulated in the ventral r1 of *Gata2*^{CKO} embryos (II/Table 1). This indicated that the rp3 domain did not produce serotonergic neurons after the inactivation of *Gata2*, although we did find Tubb3$^+$ and ELAVL3/ELAVL4 (ELAV like RNA binding protein 3 and 4, also known as HuC/D) positive post-mitotic neurons in the rV3 domain, suggesting that some neurons are still produced from these cells (II/Fig.S5) [252, 260].

Using a cDNA microarray, we were also able to determine genes that were upregulated in *Gata2*^{CKO}. One of these was *Cartpt* (*CART prepropeptide*) (II/Table 2) that was found to be expressed ectopically in the rV3 domain (II/Fig.6). Additionally, post-mitotic precursors in the rV3 domain expressed ectopically *Isl1, Nkx2-2, Nkx6-1* and *Vglut2* in *Gata2*^{CKO} (II/Fig.6). Adjacent progenitors in the ventricular zone expressed *Nkx2-2* and *Nkx6-1* (II/Fig.1). It is possible that without *Gata2* expression, post-mitotic precursors in the rV3 domain retain the progenitor-like gene expression pattern longer, as they are not directed into a serotonergic lineage. *Cartpt* encodes for CART (Cocaine- and amphetamine-regulated transcript protein), a neuropeptide normally expressed in the adult nucleus accumbens, amygdala, IPN, SNpc, Edinger-Westphal nucleus and DR [261]. *Isl1* is usually expressed by motor neurons and *Vglut2* by glutamatergic cells [262]. We did not detect any apoptotic markers in the rV3 domain at E12.5 and were not able to identify or track these ectopic cells at later stages of

development in *Gata2*^{CKO} mice (II/Fig.S5). Analysis of *Gata2*^{CKO} at E18.5 confirmed that all serotonergic neurons were lost from the DR (II/Fig.7), and that only a population of Vglut3^{+} non-serotonergic glutamatergic neurons was present, indicating that GATA2 alone is not required for the development of these cells. These results suggest that GATA2 acts as post-mitotic neuron-type selector in r1 serotonergic neurons, as without *Gata2* function the rV3-derived precursors are unable to differentiate into a serotonergic fate but rather start to express markers normally characteristic of other cell types.

5.3.3. GATA3 is responsible for the development of Sert positive subtype of serotonergic neurons in r1 (II)

We then analysed how GATA3 affects serotonergic development in r1 using *Gata3*^{CKO} embryos. At E12.5, differentiation of the dorsal Vglut3^{+} serotonergic population was not affected (II/Fig.5), but there were fewer 5-HT^{+} serotonergic neurons. Only few Sert^{+} cells were detected in the ventral rV3 domain. Similarly at E18.5, we found fewer 5-HT^{+}, Tph2^{+} and Sert^{+} neurons in the DR of *Gata3*^{CKO} embryos, while the total number of Vglut3^{+} remained constant (II/Fig.6). Unlike in *Gata2*^{CKO} embryos, ectopic expression of markers *Isl1*, *Cartpt*, *Nkx2-2*, *Nkx6-1* and *Vglut2* was not detected in the rV3 domain (II/Fig.6). These results show that contrary to the conclusions of previous studies [147], GATA3 is also needed for the development of r1 serotonergic neurons. Unlike GATA2, GATA3 does not operate as neuron-type selector in serotonergic precursors but is responsible for the development of Sert^{+} subtype serotonergic neurons in r1.

Earlier studies have suggested that GATA3 maintains serotonergic neuron-specific gene expression in the adult. Liu and colleagues inactivated *Gata3* in post-mitotic *Pet1* positive serotonergic precursors and demonstrated that it is not essential for the survival of cells but is instead required for the expression of *Tph2* and *Sert* as is evidenced by the normal number of serotonergic neurons present in the DR [140]. Similarly, we observed a loss of *Tph2* and *Sert* expression in *Gata3*^{CKO} embryos but normal numbers of total Vglut3^{+} cells at E18.5 (II/Fig.8). Therefore, it seems that in *Gata3*^{CKO} animals, post-mitotic cells belonging to the Sert^{+} serotonergic population are able to survive but simply fail to express *Sert* or *Tph2*.

5.3.4. GATA2 and GATA3 are redundantly required for the development of Vglut3^{+} non-serotonergic glutamatergic neurons in the DR

In addition to serotonergic neurons, the DR also contains a population of Vglut3^{+} non-serotonergic glutamatergic cells (II/Fig.8). As these have been relatively recently discovered [214], little is known about their development. Using *En1*$^{Cre/+}$*;R26R*^{TdTomato} and *Nkx2-2*$^{Cre/+}$*;R26R*^{TdTomato} mouse reporter lines, we found that similar to other DR neurons, Vglut3^{+} glutamatergic neurons are derived from ventral Nkx2-2^{+} progenitor cells in the rp3 of r1 (II/Fig.10 and data not shown). To determine when these cells exit the cell-cycle, we used EdU (5-ethynyl-2′-deoxyuridine) labelling technique on

Control and *Gata2*^{CKO} embryos at stages E10.5-E13.5. As serotonergic neurons are lost in *Gata2*^{CKO} embryos, while Vglut3^{+} non-serotonergic glutamatergic neurons develop normally, *Vglut3* expression can be selectively used to visualise this cell population. We observed that the majority of serotonergic neurons exited the cell-cycle between E10.5-E11.5, similar to that previously shown for r2 serotonergic neurons [198]. Vglut3^{+} non-serotonergic glutamatergic neurons became post-mitotic between E10.5-E13.5 (II/Fig.9), suggesting that some of these exit the cell-cycle a day later than serotonergic neurons. It is interesting that these neurons still express *Pet1* at E18.5 (II/Fig.10). The PET1 TF is generally considered a serotonergic specific marker that is needed in post-mitotic precursors to obtain and maintain serotonergic identity [140, 209].

In both *Gata2*^{CKO} and *Gata3*^{CKO} single mutants, Vglut3^{+} non-serotonergic glutamatergic neurons developed normally, suggesting that individually, GATA2 and GATA3 are not required for the differentiation of this cell population. We also analysed the development of these neurons in *Gata2;Gata3*^{DKO} animals at E18.5. In contrast to single mutants, in *Gata2;Gata3*^{DKO} double mutants, Vglut3^{+} non-serotonergic glutamatergic neurons were absent from the DR, indicating that GATA2 and GATA3 regulate redundantly the differentiation of these cells (II/Fig.7). Taken together, these results indicate that Vglut3^{+} non-serotonergic neurons share some similarities to serotonergic neurons such as developmental origin, time of cell-cycle exit and gene expression (*Pet1*) but require both GATA2 and GATA3 for their proper development.

5.3.5. ZFPM1 is required for the development of DRVL neurons in the DR and normal anxiety-like behaviour in mice (IV)

5.3.5.1. Zfpm1 is expressed in post-mitotic rV3 precursors and serotonergic neurons

We found that, in addition to GATA2 and GATA3, precursors in the rV3 domain also express the GATA cofactor ZFPM1, but not ZFPM2 (I/Fig.1). Analysis of *Gata2*^{CKO} and *Gata3*^{CKO} mutants showed that expression of *Zfpm1* in the rV3 domain is GATA2 but not GATA3 dependent (I/Fig.S2), suggesting that GATA2 directly or indirectly regulates the expression of *Zfpm1* in this region.

Further analysis of *Zfpm1* expression revealed that between E10.5-E12.5 in ventral r1, *Zfpm1* is expressed in post-mitotic cells of the rV3 and rV2 domains (IV/Fig.1). We had previously shown that rV3 contains separate Vglut3^{+} and Sert^{+} subpopulations of serotonergic precursors (II/Fig.2). In the rV3 domain at E12.5, we found *Zfpm1* to expressed in both populations, and co-localised with *Gata2* and *Gata3* (IV/Fig.1), indicating that it is expressed by different serotonergic populations early in development. In the neighbouring rV2 domain, *Zfpm1* was expressed by *Gad1*$^{+}$ GABAergic cells, consistent with our earlier results (IV/Fig.1 and III/Fig.4). By analysing *Zfpm1* expression at E18.5 and in the adult, we verified that *Zfpm1* expression was maintained in both serotonergic and GABAergic neurons of the DR at these stages (IV/Fig.1 and data not shown). These results show that *Zfpm1* is

expressed by serotonergic neurons early in development and also by mature neurons and, as such, may play a role in either early differentiation (like GATA2) or in the regulation of gene expression in the adult DR (like GATA3) [140].

5.3.5.2. DRVL serotonergic neurons are lost in the Zfpm1^{CKO} animals

Next, we investigated whether ZFPM1 is required for the development of serotonergic neurons or whether GATA2 and GATA3 operate independently of ZFPM1 in the rV3 domain. We used the conditional *Zfpm1* mouse mutant *Zfpm1^{CKO}* to analyse its role in their specification. In *Zfpm1^{CKO}* embryos at E12.5, expression of *Zfpm1* is lost but the expression of the serotonergic markers 5-HT, GATA2, GATA3, *Sert* and *Vglut3* is comparable to *Control* embryos. Moreover, the number of 5-HT^{+}, Vglut3^{+} and Sert^{+} neurons remained unaltered (IV/Fig.3). This indicates that ZFPM1 is not required for the early differentiation of serotonergic neurons in the r1 and that GATA2 and GATA3 act independently of ZFPM1 to regulate the development of Vglut3^{+} and Sert^{+} serotonergic precursors.

Next, we analysed expression of 5-HT, *Tph2, Sert, Vglut3* and *Pet1* in the DR of adult *Zfpm1^{CKO}* animals (IV/Fig4). The expression of these markers was generally present at the adult stage. This suggests that GATA3 operates independently of ZFPM1 to maintain gene expression in adult DR serotonergic neurons [140]. However, in these animals, expression of 5-HT, *Tph2* and *Sert* was absent from the area, where the lateral subpopulation of DR serotonergic neurons (DRVL) is normally located (Figure 14) (IV/Fig.4). Furthermore, expression of the general serotonergic marker *Pet1* was also absent from its expected location in the DRVL, demonstrating that DRVL cells are actually missing and have not just lost *Tph2* and *Sert* expression as is the case with *Gata3* mutant mice (GATA3 does not affect the expression of *Pet1*) [140]. Quantification of total 5-HT^{+} cells in the DR of *Control* and *Zfpm1^{CKO}* adult animals showed comparable numbers in both genotypes (IV/Fig.4). However, a comparison of 5-HT^{+} cells in the DR subregions DRV, DRVL, DRD and showed that while numbers remained constant in the DRV, reduced numbers were present in the DRVL area and higher numbers in the more medially located DRD of *Zfpm1^{CKO}* animals (IV/Fig.4). These results suggest that DRVL cells are misplaced to the DRD subregion in the *Zfpm1^{CKO}*. Supportive of this hypothesis is the observation of *BCL11B* expression differences between serotonergic neurons in *Control* and *Zfpm1^{CKO}* animals. In *Control* animals at E18.5, BCL11B^{+} serotonergic neurons can be found exclusively in the DRVL subregion, while in *Zfpm1^{CKO}* animals, DRVL cells are missing from their correct location and BCL11B^{+} serotonergic neurons are found ectopically in the DRD instead (IV/Fig.4).

Interestingly, a similar phenotype, where DRVL are absent but more serotonergic neurons are positioned in the midline of DR, have been observed in mouse mutants where Reelin signalling has been disrupted such as seen in the *Reelin* (RELN) mutant and double mutants of the RELN receptors *Lrp8* (*Low density lipoprotein receptor-related protein 8*, also known as *ApoER2*) and *Vldlr* (*Very low-density lipoprotein receptor*) [263]. The role of Reelin signalling in the migration and correct positioning of the

neurons has been previously shown in the cortex (to achieve correct layering of neurons), SNpc (for the lateral migration of dopaminergic neurons) and the cerebellum (for the migration of Purkinje cells during Purkinje plate formation) [264-266]. Additional experiments are needed to determine whether ZFPM1 is involved in the expression of Reelin pathway components.

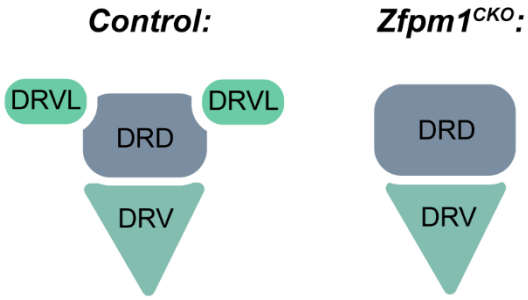

Figure 14. Inactivation of *Zfpm1* in early development of r1-derived serotonergic neurons causes a phonotype where laterally positioned DRVL serotonergic neurons are missing from DR, whereas the number of serotonergic neurons in medially positioned DRD subregion of DR is increased.

5.3.5.3. Decreased serotonin levels in the Zfpm1^{CKO} mice

DR has collateral innervation to majority of rostral brain structures, some of which have been implicated in the regulation of anxiety, such as the amygdala, dorsal midbrain, prefrontal cortex and hippocampus [222, 223, 267]. To further characterise this, we determined the concentrations of serotonin and its metabolite 5-HIAA (5-hydroxyindoleacetic acid) in prefrontal cortex, hippocampus and dorsal midbrain (the amygdala was excluded due to dissection challenges) using HPLC (High-Performance Liquid Chromatography). In *Zfpm1^{CKO}* animals, we detected lower concentrations of serotonin and 5-HIAA in the prefrontal cortex and the hippocampus, whereas their concentrations in the dorsal midbrain remained comparable to *Control* animals (IV/Fig.5 and IV/Table S1). This suggests that serotonergic innervation of these brain structures is altered in the *Zfpm1^{CKO}* animals.

5.3.5.4. The behaviour of Zfpm1^{CKO} animals suggests increased anxiety

We demonstrated that *Zfpm1^{CKO}* mice lack DRVL serotonergic neurons, have increased numbers of serotonergic neurons in the DRD as well as lower serotonin levels in the prefrontal cortex and hippocampus. These regions have been shown to differentially regulate anxiety behaviour [217, 218, 268]. Due to this reason, we decided to analyse anxiety-like behaviour in these mice. In the elevated plus maze, *Zfpm1^{CKO}* mice did not explore open arms as frequently as *Control* animals, demonstrating an increased avoidance of open spaces which is indicative of increased anxiety-like behaviour (IV/Fig.6). However, in the forced swim test, *Zfpm1^{CKO}* animal mobility was unaltered compared to that of *Control* mice (IV/Fig.7). These results show that *Zfpm1^{CKO}* mice have increased anxiety-like behaviour but normal depression-like behaviour.

Previous experiments with specific mouse lines, where the survival of serotonergic neurons (*Pet1*$^{Cre/+}$;*Lmx1b*$^{flox/flox}$ and *Pet1*^{CreERT2};*Rosa26*^{DTA}) or the synthesis of serotonin is impaired (*Pet1*$^{Cre/+}$;*Tph2*$^{flox/flox}$), have helped shed light on how serotonin concentrations in the brain affect anxiety. It has been shown that overall lower concentrations of serotonin causes reduced anxiety-like behaviour during the elevated plus maze but does not affect depression as demonstrated by forced swim test [224, 225, 269]. This is in contrast to *Zfpm1*^{CKO} animals, where we observe elevated anxiety-like behaviour. These differences could be explained by the fact that *Zfpm1*^{CKO} mice have not lost all serotonergic neurons but rather undergo a change in their distribution within the DR. They may also be due to changes in projections to rostral brain structures such as the hippocampus and prefrontal cortex. Interestingly, pharmacological depletion of serotonin in the ventral hippocampus increases anxiety-like behaviour in the elevated plus maze test [267]. Furthermore, *Zfpm1*^{CKO} animals have an increased number of serotonergic neurons in the DRD area (IV/Fig.4). It has been shown that optogenetic activation of DRD neurons (that project to amygdala) causes increased anxiety-like behaviour in the elevated plus maze [217]. Higher serotonergic input from DRD to amygdala in *Zfpm1*^{CKO} would additionally explain their higher anxiety-like behaviour, however the projection targets of ectopic DRD neurons needs to be confirmed by additional studies.

5.3.5.5. Zfpm1 mutants show enhanced fear conditioning that is alleviated by SSRI treatment

Since the amygdala, prefrontal cortex, hippocampus and DR are also involved in the regulation of fear learning, we analysed *Zfpm1*^{CKO} animals using contextual fear conditioning, where a specific environment becomes associated with an aversive foot-shock [217, 226-228]. In this test, *Zfpm1*^{CKO} animals showed increased freezing in three subsequent post-shock days, indicating increased acquisition and reduced extinction of contextual fearful memories (IV/Fig.7). This behavioural phenotype might be caused by an excessive number of DRD serotonergic neurons in *Zfpm1*^{CKO} animals (IV/Fig.4). Earlier experiments have demonstrated that optogenetic activation of DRD cells causes similar increased freezing in cued fear conditioning [217]. Additionally, mice that have reduced serotonin concentrations in the brain also exhibit increased freezing in the contextual fear conditioning [224, 225].

As, *Zfpm1*^{CKO} mutants exhibited decreased serotonin levels and displayed increased freezing during contextual fear conditioning tests, we investigated the effects of chronic administration of fluoxetine , a selective serotonin reuptake inhibitor (SSRI), on their behaviour by carrying out contextual fear conditioning and subsequent fear reinstatement experiments. Like previously shown, chronic fluoxetine (flx) administration lowered freezing of *Control* (flx) animals at the fear reinstatement phase compared to *Control* (water treated) mice (IV/Fig.7) [270]. However, chronic fluoxetine administration decreased the freezing of *Zfpm1*^{CKO} (flx) mice, in all stages of contextual fear conditioning and also at the fear reinstatement phase compared to the *Zfpm1*^{CKO} (water treated) and had decreased it to similar

level as was seen in the *Control* (water treated) group (IV/Fig.7). These results show how chronic fluoxetine administration is able to reduce increased fear memory in *Zfpm1*^{CKO} animals.

Chronic administration of SSRIs is used to alleviate symptoms of anxiety and depression in patients [213, 271]. SSRIs are antidepressants that increase the concentration of extracellular serotonin by binding to the serotonin transporter SERT and inhibiting the reuptake of serotonin to presynaptic neurons [272]. SSRIs are effective in altering the anxiety-and depression-like behaviour of rodents and also affect the expression of conditioned fear [273, 274]. For example, acute administration of fluoxetine in mice decreases immobility in the forced swim test suggesting an antidepressant effect of fluoxetine [275]. Moreover, mice that have received chronic fluoxetine treatment display normal fear acquisition and expression during contextual fear conditioning but have decreased freezing in subsequent fear renewal (fear memory is recovered when animal is reexposed to context) and fear reinstatement (fear memory is reinstated when animal receives foot-shocks again in the same context) that are thought to be caused by changes in neuronal plasticity that erases conditioned fear after fear extinction [270]. This behavioural change in *Zfpm1*^{CKO} animals is presumably caused by higher extracellular serotonin levels in the brain after fluoxetine treatment. Earlier experiments with *Pet1*$^{Cre/+}$;*Lmx1b*$^{flox/flox}$ and *Pet1*^{CreERT2};*Rosa26*^{DTA} mouse lines have shown that reduced concentration of serotonin in brain causes increased contextual fear memory indicating that normal serotonin levels are important for normal fear memory [224, 225]. Additionally, chronic fluoxetine treatment has been shown to impair the formation of fear memories during contextual fear conditioning, suggesting that fluoxetine treated *Zfpm1*^{CKO} animals fail to form new fear memories after aversive events [276].

6. CONCLUDING REMARKS

The anterior brainstem is composed of numerous GABAergic and serotonergic nuclei that play critical roles in the regulation of various essential CNS functions. Therefore, gaining a clearer understanding of the mechanisms underlying their development is crucial. In this study we focused on the ventral area of r1 with the goal of advancing knowledge of the unique cell types generated from the region as well as elucidating the molecular mechanisms that determine their developmental fate. We were able to show that embryonic ventral r1 contains an abundance of diverse cell subtypes that give rise to several distinct nuclei in the ventral midbrain and hindbrain during later developmental stages. Furthermore, we verified the developmental origin of these groups within r1 and identified novel markers for these subtypes and nuclei. These possess great potential as tools to be used in future studies.

To understand how neuronal heterogeneity is generated, we studied the role of GATA TFs (GATA2 and GATA3) and their co-regulators TAL1, TAL2, ZFPM1 and ZFPM2 in development of ventral r1 cell groups. We revealed that GATA TFs and their co-regulators are important in r1 to achieve neuronal diversity as they are required differently in serotonergic, GABAergic and small populations of glutamatergic precursors.

The findings in this thesis work give insights into how cell identity is controlled in the CNS, how GATA TFs regulate it, and how GATA TFs can operate independently of their cofactors to specify the developmental program of some neuron subtypes. Furthermore, analysis of *Zfpm1*^{CKO} mice has uncovered how changes in DR serotonergic subtypes can cause increased anxiety-like behaviour and how SSRI administration can alleviate this phenotype.

The main conclusions are:

Conclusion 1:

The developing ventral r1 contains at least three progenitor domains that give rise to serotonergic (rV3), GABAergic and glutamatergic precursors (rV2), and oligodendrocytes (rvMN). Embryonic ventral r1 contains cell populations that originate there or are derived from cells that migrate there from dorsal r1. GABAergic rV2 precursors give rise to pSNpr, RMTg and VTg neurons, whereas glutamatergic rV2 precursors produce IPN and LDTg neurons.

Future studies should be directed at determining whether these progenitor domains consist of even smaller subregions dorso-ventrally and anterior-posteriorly that potentially contain other novel progenitors that specify distinct cell populations.

Conclusion 2:

In the rV2 domain, *Tal1* and *Gata2*/*Gata3* act redundantly as neuron-type selector genes to determine a GABAergic over glutamatergic fate of post-mitotic precursors, and are required for development of rV2-derived GABAergic nuclei pSNpr, RMTg and VTg.

Additional studies should investigate whether expression of *Tal1* and *Gata2*/*Gata3* in early post-mitotic precursors in the rV2 domain is dependent on Notch signalling similar to the spinal cord [153].

Conclusion 3:

GATA cofactors *Zfpm1* and *Zfpm2* are expressed in early rV2 GABAergic precursors. ZFPM factors are not required for GATA function during GABAergic vs. glutamatergic fate choice. However, ZFPM2 is required for later development of GABAergic neurons in the pSNpr, RMTg and VTg.

Future studies should analyse GABAergic development further in $Zfpm2^{CKO}$ animals to determine what causes the loss of rV2-derived GABAergic neurons at later stages of development (failure of migration, change of subtype identity or inability to survive).

Conclusion 4:

In the rV3 domain, *Gata2* functions as a neuron-type selector gene required for the serotonergic fate of post-mitotic precursors. However, *Gata3* is essential for the development of a subtype ($Sert^{+}$) of r1-derived serotonergic neurons. Moreover, *Gata2* and *Gata3* are functionally redundant during the development of a glutamatergic neuron subtype ($Vglut3^{+}$) in the DR (DRDsh). These DR glutamatergic neurons are related to DR serotonergic neurons by their developmental origins, regulatory mechanisms, and gene expression characteristics.

Conclusion 5:

GATA cofactor *Zfpm1* is expressed in post-mitotic serotonergic precursors, but early differentiation of serotonergic neurons is not affected by its loss. *Zfpm1* is required for the correct distribution of serotonergic neuron subtypes in the DRVL and DRD, as well as for normal serotonin levels in the brain. Mutant animals have increased anxiety-like behaviour and elevated fear memory that is reduced with chronic fluoxetine treatment.

Subsequent studies should analyse how *Zfpm1* affects the migration/positioning of DRVL neurons, by possibly regulating the expression of Reelin pathway components. Additionally, the projection targets of ectopic DRD neurons in $Zfpm1^{CKO}$ animals should be determined so as to better understand how abnormal distribution of serotonergic neurons leads to elevated anxiety-like behaviour.

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