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RESEARCH ARTICLE

Gata2 and Gata3 regulate the differentiation of serotonergic and glutamatergic neuron subtypes of the dorsal raphe

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ABSTRACT

Serotonergic and glutamatergic neurons of the dorsal raphe regulate many brain functions and are important for mental health. Their functional diversity is based on molecularly distinct subtypes; however, the development of this heterogeneity is poorly understood. We show that the ventral neuroepithelium of mouse anterior hindbrain is divided into specific subdomains giving rise to serotonergic neurons as well as other types of neurons and glia. The newly born serotonergic precursors are segregated into distinct subpopulations expressing vesicular glutamate transporter 3 (*Vglut3*) or serotonin transporter (*Sert*). These populations differ in their requirements for transcription factors Gata2 and Gata3, which are activated in the post-mitotic precursors. Gata2 operates upstream of Gata3 as a cell fate selector in both populations, whereas Gata3 is important for the differentiation of the *Sert*$^{+}$ precursors and for the serotonergic identity of the *Vglut3*$^{+}$ precursors. Similar to the serotonergic neurons, the *Vglut3*-expressing glutamatergic neurons, located in the central dorsal raphe, are derived from neural progenitors in the ventral hindbrain and express *Pet1*. Furthermore, both Gata2 and Gata3 are redundantly required for their differentiation. Our study demonstrates lineage relationships of the dorsal raphe neurons and suggests that functionally significant heterogeneity of these neurons is established early during their differentiation.

KEY WORDS: Serotonergic neuron, Glutamatergic neuron, Dorsal raphe, Rhombomere 1, Gata2, Gata3

INTRODUCTION

Serotonin modulates activity in essentially all regions of the brain, and imbalance in the serotonergic systems has been associated with complex psychiatric disorders including depression, anxiety, obsessive compulsive behaviour, impulsivity, autism and sleep disorders (Deneris and Wyler, 2012). Despite the wide range of functions, the source of serotonin in the brain is in relatively few serotonergic neurons. In the hindbrain, the serotonergic neurons form two main clusters – rostral and caudal (Dahlstroem and Fuxe, 1964; Alonso et al., 2013; Okaty et al., 2015). The rostral cluster includes subgroups of dorsal and median raphe nuclei and sends mostly ascending projections to the forebrain and midbrain, modulating higher brain functions, whereas the caudal cluster has primarily descending connections to the spinal cord.

Recent studies have started to unravel molecular and functional heterogeneities among the serotonergic neurons. Transcriptome analyses have mapped the differences in gene expression between and within the rostral and caudal clusters (Wylie et al., 2010; Okaty et al., 2015). In addition, the dorsal and median raphe serotonergic neurons display heterogeneity in their serotonin autoreceptor (Htr1a) and serotonin transporter (Sert; also known as Slc6a4) expression, electrophysiological properties, axonal morphology and susceptibility to neurotoxins (Mamounas et al., 1991; Crawford et al., 2010; Calizo et al., 2011; Kiyasova et al., 2013). Serotonergic neurons in the dorsal raphe are also thought to differ in their use of co-neurotransmitters (Fu et al., 2010). These include glutamate as vesicular glutamate transporter 3 (Vglut3; Slc17a8) is produced in a subset of dorsal raphe serotonergic neurons (Hioki et al., 2010). In addition to the Vglut3^{+} serotonergic neurons, a specific subgroup of non-serotonergic Vglut3^{+} cells is located in the central dorsal raphe. Furthermore, recent studies demonstrated the importance of glutamatergic neurotransmission from the dorsal raphe to ventral tegmental area (VTA) in the regulation of motivation and reward processing (Hioki et al., 2010; Liu et al., 2014; McDevitt et al., 2014; Qi et al., 2014; Sego et al., 2014). Developmental mechanisms underlying the neuronal heterogeneity in the dorsal raphe are still poorly understood.

The serotonergic nuclei arise from different embryonic hindbrain segments (Cordes, 2005; Kiyasova and Gaspar, 2011; Deneris and Wyler, 2012). The dorsal raphe develops from a single anterior hindbrain segment, rhombomere 1 (r1), whereas the median raphe neurons originate from r1, r2 and r3 (Jensen et al., 2008). The serotonergic neurons develop adjacent to a ventral neuroepithelial region marked by a homeodomain (HD) transcription factor (TF) Nkx2-2 (Briscoe et al., 1999). Misexpression of Nkx2-2 in the dorsal r1 leads to induction of ectopic serotonergic neurons (Craven et al., 2004). However, Nkx2-2 is not required for serotonergic neuron development in the r1 (Briscoe et al., 1999; Craven et al., 2004; Jensen et al., 2008). This has been suggested to be due to redundancy of Nkx2-2 with a related TF, Nkx2-9 (Briscoe et al., 1999; Pattyn et al., 2003). The r1 serotonergic progenitors express and require another HD TF, Nkx6-1, in the chicken (Craven et al., 2004; Deneris and Wyler, 2012). The proliferative serotonergic neuron progenitors also express *FoxA2*, as well as the proneural gene *Ascl1*, which are important for serotonergic neuron development (Pattyn et al., 2004; Norton et al., 2005; Jacob et al., 2007). However, it is unclear whether these TFs contribute to patterning of the ventral r1 progenitors to give rise to distinct serotonergic neuron subtypes.

Other TFs are activated and trigger the expression of a serotonergic neuron-specific gene battery after the cell cycle exit of the precursors. These include Gata2, Gata3, Pet1, Lmx1b and Insm1 (Hendricks et al., 1999, 2003; van Doorninck et al., 1999;

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Cheng et al., 2003; Ding et al., 2003; Craven et al., 2004; Pattyn et al., 2004; Jacob et al., 2009). In the r1, both Gata2 and Gata3 are expressed in GABAergic as well as serotonergic precursors. In contrast to the r1 GABAergic precursors, which retain *Gata3* expression in the absence of Gata2, *Gata3* expression is lost in the serotonergic precursors in *Gata2* mutant embryos (Kala et al., 2009). The expression of *Pet1*, *Lmx1b* and other functional serotonergic neuron markers are also absent from the *Gata2* mutant r1 (Craven et al., 2004; Kala et al., 2009). Consistently, Gata2 directly regulates the expression of Pet1, a serotonergic neuron-specific TF that binds to regulatory regions of serotonergic gene battery, including *Tph2*, *Sert* and *Htr1a* (Krueger and Deneris, 2008). Thus, Gata2 appears to be high in the gene regulatory hierarchy activated in the postmitotic serotonergic neuron precursors. In contrast to *Gata2*, inactivation of *Gata3* leads to a modest reduction in the numbers of serotonergic neurons in the perinatal anterior hindbrain (Pattyn et al., 2004; Liu et al., 2010).

Some observations indicate heterogeneity in the molecular regulation of development in the distinct populations of serotonergic neurons. For example, in *Pet1* mutants, about 30% of the serotonergic neurons are spared and the remaining neurons have distinct projection targets in the brain (Hendricks et al., 2003; Kiyasova et al., 2011). Thus, variation in the regulatory TFs during early development might translate into distinct subtypes of serotonergic neurons. However, the mechanisms underlying the cell type heterogeneity, especially within the individual raphe nuclei, remain unclear.

Here, we show that the neural cell populations derived from the ventral r1 give rise to the entire cell type complement in the dorsal raphe. We used genetic fate mapping to demonstrate that, in addition to some other types of neurons and glia, serotonergic precursors are derived from the *Nkx2-2*-expressing progenitors in the ventral r1. These serotonergic precursors are sorted into molecularly distinct subgroups from the earliest stages of their development, contribute to different serotonergic neuron subtypes of the dorsal raphe, and differ in their pattern of expression of Gata2 and Gata3. We show by loss-of-function analyses that there are unique requirements for Gata2 and Gata3 in the development of the serotonergic neuron precursor populations. Finally, we show that the glutamatergic neurons of the dorsal raphe are developmentally related to serotonergic neurons.

RESULTS

Refined map of the progenitor domains in mouse ventral r1

Studies of Nkx2-2 expression and function suggest that serotonergic neurons are derived from the Nkx2-2-positive progenitors in the ventral r1. To understand further the homeodomain TF codes and neuronal populations within the Nkx2-2 domain, we compared the expression of Nkx2-2, Nkx2-9, Nkx6-1 and Nkx6-2 in the mouse ventral r1 at embryonic day (E) 10.5-E12.5. Nkx2-9 has been suggested to cooperate with Nkx2-2 to pattern the serotonergic progenitors (Briscoe et al., 1999). We observed expression of *Nkx2-9* in a narrow area in the dorsal Nkx2-2^{+} domain, thus dividing the Nkx2-2 progenitors into two domains, which we named rp3 and rpvMN (rhombencephalic progenitor domain 3 and rhombencephalic visceral motor neuron progenitor domain, respectively; nomenclature adopted from the spinal cord) (Fig. 1A-C,E-G) (Briscoe et al., 2000; Lahti et al., 2016). In chicken, Nkx6-1 is expressed in serotonergic neuron progenitors and can induce serotonergic neuron differentiation cooperatively with Nkx2-2 (Craven et al., 2004). We observed expression of Nkx6-1 in E10.5-E12.5 mouse rp3 and rpvMN but, in contrast to chicken embryos, in which strong Nkx6-1 expression was associated with serotonergic neurogenesis, the level of Nkx6-1 expression was low in the mouse serotonergic rp3 domain compared with the GABAergic/glutamatergic rp2 domain, especially at E11.5-E12.5 (Fig. 1I-K; Fig. S7B,E) (Lahti et al., 2016). Interestingly, the level of Nkx6-1 expression in the rp3 progenitors appeared to be graded, increasing towards the dorsal boundary of the Nkx2-2 expression. We also mapped the expression of a related TF, Nkx6-2, along the progenitor domains in the ventral r1. Similar to Nkx6-1, Nkx6-2 displayed a dorsally increasing gradient, but its expression was more restricted to rpvMN and dorsal rp3 (Fig. S1A-C).

We next used genetic fate mapping in combination with known cell type markers to characterise further the cell lineages arising from the rp3 and rpvMN progenitor domains positive for Nkx2-2. At E10.5-E11.5, precursors expressing the motor neuron marker Islet1 were detected adjacent to both rp3 and rpvMN domains in the anterior r1 (Fig. 1M,N; data not shown). In the *Nkx2-2^{Cre};ROSA26^{TdTomato}* mice, in which Cre-mediated recombination in *Nkx2-2*-expressing cells permanently labels them with RFP expression, some Islet1-positive cells were RFP positive at E10.5 (Fig. 1Q,Q′). However, *Nkx2-2^{Cre}* appeared to mediate only a mosaic recombination as 20% and 7% of the Nkx2-2-positive progenitors lacked RFP expression at E10.5 and E12.5, respectively (Fig. 1Q, arrow; data not shown). Nevertheless, we can conclude that at least some Islet1-positive cells are generated in the Nkx2-2 domain. Although the activity of the *Nkx2-2* locus-driven Cre recombinase may not be early, strong or broad enough to label all Islet1-expressing precursors, we cannot exclude the possibility that some of the Islet^{+} cells are born outside the Nkx2-2 domain. Similar to E10.5, mosaic recombination by the *Nkx2-2^{Cre}* was detected in Islet1^{+} trochlear neurons at E18.5 (data not shown).

At E12.5, serotonergic neuron markers 5-hydroxytryptamine (5-HT) and *Pet1* were detected adjacent to the Nkx2-2-positive progenitors, consistent with earlier studies (Fig. 1D; Fig. 5A,I). Furthermore, the *Pet1*-expressing precursors were efficiently labelled by RFP in *Nkx2-2^{Cre};ROSA26^{TdTomato}* embryos at E12.5, demonstrating their origin in the Nkx2-2-expressing progenitors (Fig. 1S,S′). Interestingly, only the Nkx2-2^{+}Nkx2-9^{-} rp3 region appeared to give rise to serotonergic precursors. Instead, next to the adjacent Nkx2-2^{+}Nkx2-9^{+} rpvMN, we observed precursors expressing oligodendrocyte markers Olig2, *Sox10* and Pdgfra (Fig. 1H,O; data not shown). This oligodendrocyte population was also labelled in the *Nkx2-2^{Cre};ROSA26^{TdTomato}* embryos, supporting their origin from the Nkx2-2-positive progenitors (Fig. 1R,R′), consistent with earlier studies in more posterior rhombomeres (Vallstedt et al., 2005). The Nkx2-2 domain was bordered by the rp2 region, which gives rise to GABAergic and glutamatergic neurons (Fig. 1L,P) (Lahti et al., 2016).

Thus, we find that the progenitors in the ventral r1 can be divided into smaller subdomains that generate different neural cell types, including serotonergic neurons and oligodendrocytes. The results of our TF expression analysis and genetic fate mapping are summarised in Fig. 1T.

Post-mitotic serotonergic neuron subtypes are segregated early in development

In the adult brain, functionally and molecularly different serotonergic neuron subgroups can be distinguished by the expression of *Vglut3* and *Sert* (Hioki et al., 2010). To understand when and how this diversity is established, we first analysed *Vglut3* and *Sert* expression in comparison with the broadly expressed early

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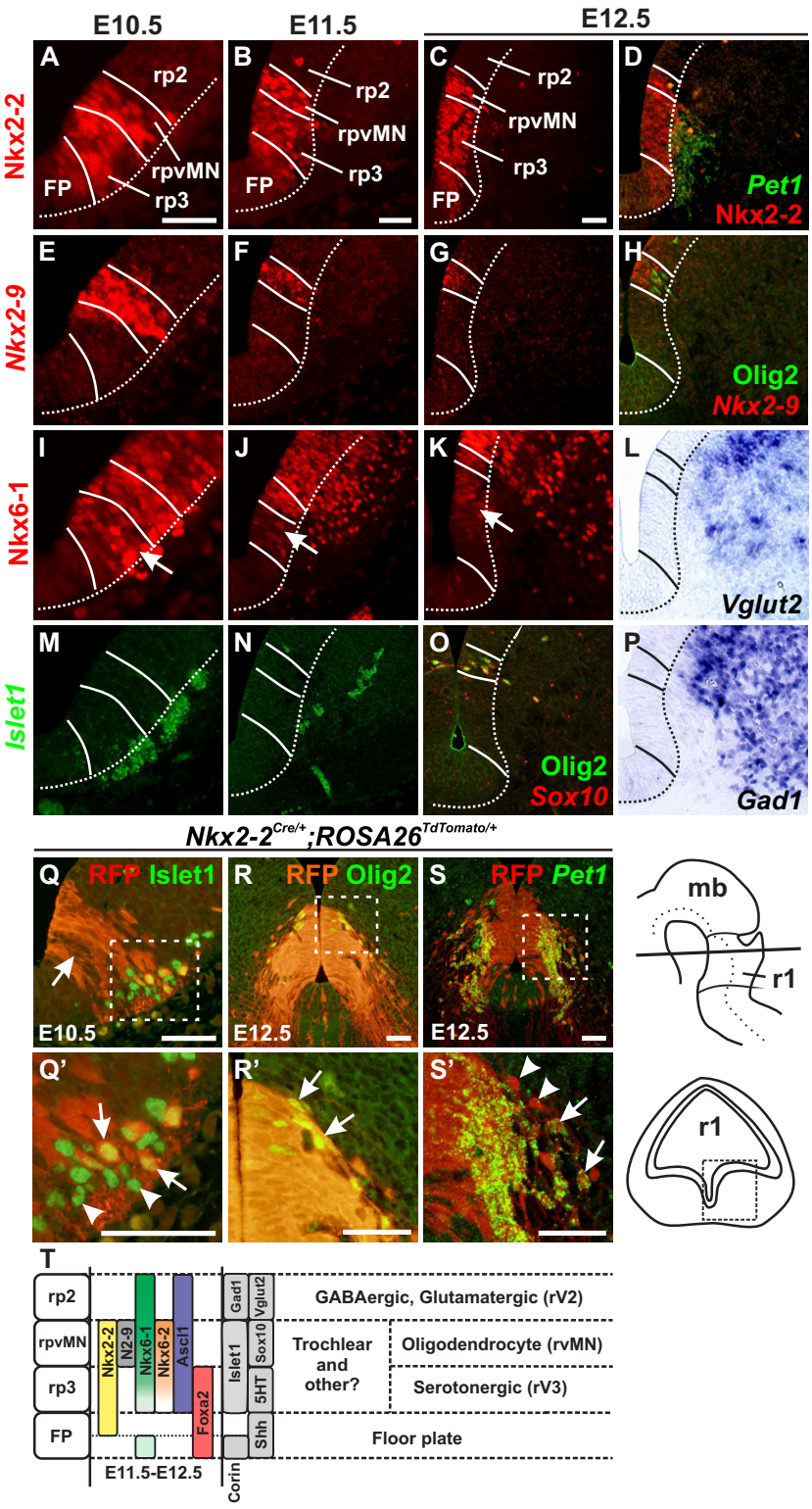

Fig. 1. Progenitor domains producing neuronal variety in the ventral r1. (A-C) Nkx2-2 IHC on ventral r1 at E10.5-E12.5 (sectioning plane shown bottom right). (D) Nkx2-2 IHC and *Pet1* ISH. (E-G) *Nkx2-9* ISH on sections parallel to those in A-C. (H) *Nkx2-9* ISH and Olig2 IHC. (I-K) Nkx6-1 IHC at E10.5-E12.5; arrows indicate graded expression in rp3. (L) *Vglut2* ISH. (M,N) *Islet1* ISH on sections parallel to those in A,B. (O) *Sox10* ISH and Olig2 IHC. (P) *Gad1* ISH. (Q-S′) Sections of *Nkx2-2*$^{Cre/+}$;*ROSA26*$^{TdTomato/+}$ embryos. (Q,Q′) RFP and Islet1 IHC on ventral r1 at E10.5; boxed region in Q is shown at higher magnification in Q′. Arrow in Q indicates mosaic expression of RFP among progenitors. Arrows in Q′ mark RFP$^+$ Islet1$^+$ cells, arrowheads mark cells that express Islet1 but not RFP. (R,R′) RFP and Olig2 IHC at E12.5; boxed region in R is shown at higher magnification in R′. Arrows mark RFP$^+$Olig2$^+$ cells. (S,S′) RFP IHC and *Pet1* ISH at E12.5; boxed region in S is shown at higher magnification in S′. Arrows indicate RFP$^+$ *Pet1*$^+$ cells, arrowheads indicate RFP+ *Pet1*$^-$ cells. (T) Ventral r1 progenitor domains producing different types of neural progeny. Many of the TFs shown are important for serotonergic neuron development in the mouse r1 or posterior rhombomeres (Briscoe et al., 1999; Pattyn et al., 2004; Jacob et al., 2007; Jensen et al., 2008). FP, floor plate; mb, midbrain; rV2,3, rhombencephalic V2, V3 precursor domain; rvMN, rhombencephalic vMN precursor domain. Scale bars: 50 µm.

serotonergic marker *Pet1*. Interestingly, the expression of *Vglut3* and *Sert* was already segregated to discrete dorsal and ventral populations, respectively, of *Pet1*$^+$ precursors at E11.5-E12.5 (Fig. 2A-D; Fig. S2A-D). Separate *Vglut3*- and *Sert*-expressing populations were also observed in different parts of the dorsal and median raphe complex later in embryonic development at E14.5, E16.5 and E18.5 as well as in the adult brain (Fig. 2F-L; Fig. S2E-S). However, at E16.5, E18.5 and in the adult dorsal raphe, some cells were also found to co-express these markers, especially in the medial part of the dorsal raphe (Fig. 2F,E,H,J,K,M). *Sert*$^+$ precursors apparently produce only serotonergic neurons, whereas the *Vglut3*$^+$ precursors may give rise to either *Vglut3*$^+$ serotonergic neurons using glutamate as a co-neurotransmitter or *Vglut3*$^+$ glutamatergic neurons lacking serotonin biosynthesis (Hioki et al., 2010). From E11.5 onwards, some of the *Vglut3*$^+$ cells also expressed *Tph2*, showing that at least some of them become

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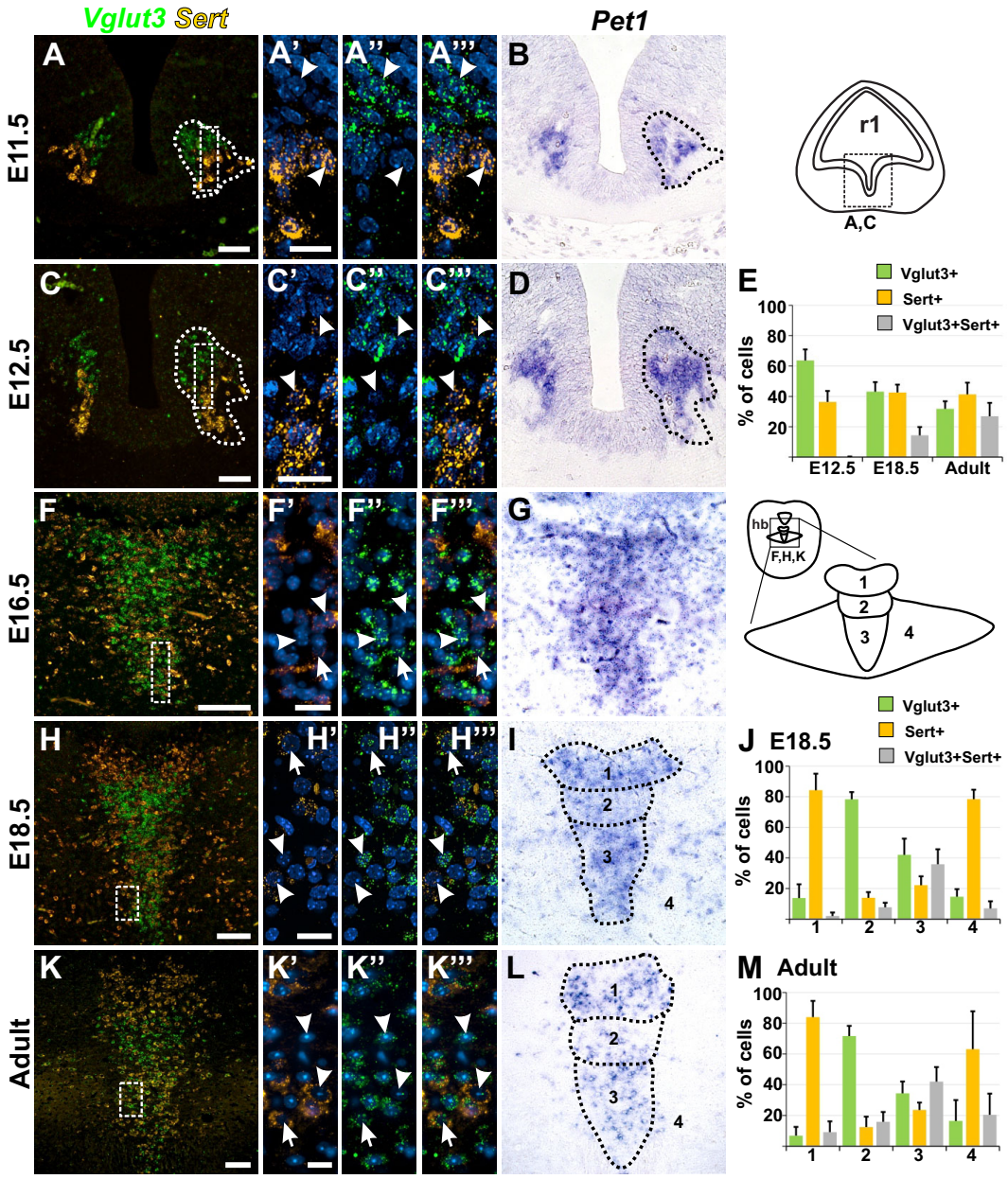

Fig. 2. *Vglut3*- and *Sert*-expressing populations in the developing and adult dorsal raphe. (A-A‴,C-C‴,F-F‴,H-H‴,K-K‴) Combined *Vglut3* and *Sert* ISH at E11.5, E12.5, E16.5 and E18.5 and in an adult brain; boxed regions are shown at higher magnification in A′-A‴,C′-C‴,F′-F‴,H′-H‴,K′-K‴. Arrows indicate double-positive, arrowheads single-positive cells. (B,D,G,I,L) *Pet1* ISH on sections parallel to those in A,C,F,H,K. (E,J,M) Relative amounts of *Vglut3*$^+$, *Sert*$^+$ and *Vglut3*$^+$*Sert*$^+$ cells at E12.5, E18.5 and in the adult, and their regional distribution in the dorsal raphe at E18.5 and in adult. Regions analysed are indicated in the schematics and in I,L. *Pet1*-expressing area is outlined in A-D. Bars show mean±s.d. hb, hindbrain. Scale bars: 20 µm (a′-k′), 50 µm (A,C), 100 µm (F,H,K).

serotonergic (Fig. S3A-C). In summary, our results suggest that *Vglut3* and *Sert* are expressed in largely complementary subgroups of *Pet1*$^+$ post-mitotic precursors in the early stage of their differentiation. Later, neurons in the dorsal raphe express either *Sert* or *Vglut3* or both of them. These subtypes are differently distributed in the dorsal raphe complex.

Because we had observed graded expression of Nkx6-1 and Nkx6-2 along the serotonergic progenitor domain (Fig. 1I-K; Fig. S1A-C), we investigated whether this heterogeneity correlated with the above-mentioned cell types. Indeed, *Vglut3*-expressing precursors were located mostly next to the dorsal rp3 progenitors that are positive for Nkx6-1 and Nkx6-2, whereas *Sert* expression was detected more ventrally (Fig. 3A,B; data not shown). Thus, although the low level of Nkx6-1 and Nkx6-2 expression makes outlining of the exact rp3 subregions difficult, their expression in the progenitors spatially correlates with the development of distinct *Vglut3*$^+$ and *Sert*$^+$ precursor populations.

Expression of Gata2 and Gata3 during serotonergic neuron differentiation

Gata2 and Gata3 are important regulators of the development of serotonergic neurons and are differentially required in the rostral and caudal serotonergic neuron complexes (van Doorninck et al., 1999; Pattyn et al., 2004). To understand whether previously uncharacterised heterogeneity in Gata TF expression correlates with the development of the serotonergic neuron subgroups within the r1,

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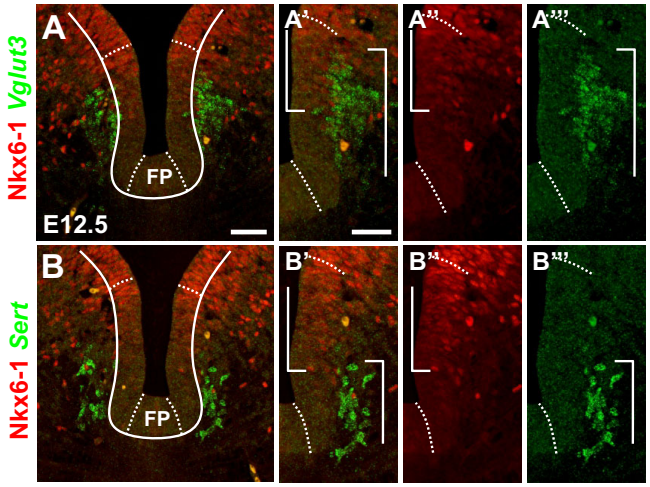

Fig. 3. Comparison of Nkx6-1 expression in the rp3 progenitors to the *Vglut3*$^{+}$ and *Sert*$^{+}$ precursors. (A-A‴) *Vglut3* ISH and Nkx6-1 IHC at E12.5; higher magnifications shown in A′-A‴. (B) *Sert* ISH and Nkx6-1 IHC at E12.5; higher magnifications shown in B′-B‴. Dotted lines mark Nkx2-2 expression range on parallel sections (not shown). Brackets indicate expression area of analysed genes. FP, floor plate. Scale bars: 50 µm.

we analysed the expression of Gata2 and Gata3 in the *Vglut3*$^{+}$ and *Sert*$^{+}$ precursor populations. In the rp3, Gata2 and Gata3 were first detected at E10.5 when serotonergic neuron precursors start exiting the cell cycle (Fig. S4A-I). At E12.5, Gata2 and Gata3 were broadly expressed in the ventral r1 including the serotonergic precursors (Fig. 4A-A‴). However, we also observed some differences in the staining intensities of Gata2 and Gata3 along the *Vglut3*- and *Sert*-expressing precursor populations. Whereas Gata3 was detected in both subgroups at approximately the same intensity, Gata2 expression seemed stronger in the early *Vglut3*$^{+}$ precursors compared with the *Sert*$^{+}$ precursors (Fig. 4B′-E‴). In the dorsal raphe at E16.5, cells were heterogeneous for Gata2 and Gata3 expression and included all Gata2^{+}, Gata3^{+} and Gata2^{+}Gata3^{+} subtypes. However, we detected both of these factors in both *Vglut3*$^{+}$ and *Sert*$^{+}$ subgroups (Fig. 4F-J‴). Thus, although early in development the *Vglut3*$^{+}$ and *Sert*$^{+}$ serotonergic precursors show differences in the relative amounts of Gata2 and Gata3 expression, both cell types express them later in the dorsal raphe.

Gata2 is not required for the production of neuronal precursors but is necessary for activation of serotonergic neuron-specific gene expression

Although Gata TFs, especially Gata2, are known to be involved in the development of serotonergic neurons, their exact role in this process is still unclear, largely owing to early embryonic lethality of *Gata2* and *Gata3* null mutant mice (Bresnick et al., 2010). To analyse the functions of Gata2 and Gata3 in serotonergic neuron development in the r1, we conditionally inactivated *Gata2* (*Gata2*^{flox} allele) and *Gata3* (*Gata3*^{flox}) using the *En1*^{Cre} allele, which drives efficient Cre-mediated recombination in the midbrain and r1 as early as E8.5 (Trokovic et al., 2003). We detected no Gata2 or Gata3 expression in *En1*^{Cre};*Gata2*$^{flox/flox}$ (*Gata2*^{En1cko}) or *En1*^{Cre};*Gata3*$^{flox/flox}$ (*Gata3*^{En1cko}) embryos, respectively, at E11.5-E12.5 (Fig. S4J-S).

First, we investigated whether Gata2 is required for the production or survival of post-mitotic serotonergic neuron precursors. We observed no apparent changes in the expression of postmitotic neuron markers [Tuj1 (also known as Tubb3) and HuC/D (also known as Elavl3/4)], number of apoptotic cells or morphological appearance of the E12.5 ventral r1 of *Gata2*^{En1cko} mutants compared with controls (Fig. S5A-F). However, the post-mitotic precursors produced in the Nkx2-2 domain of *Gata2*^{En1cko} embryos had lost both general and subtype-specific aspects of the serotonergic neuron

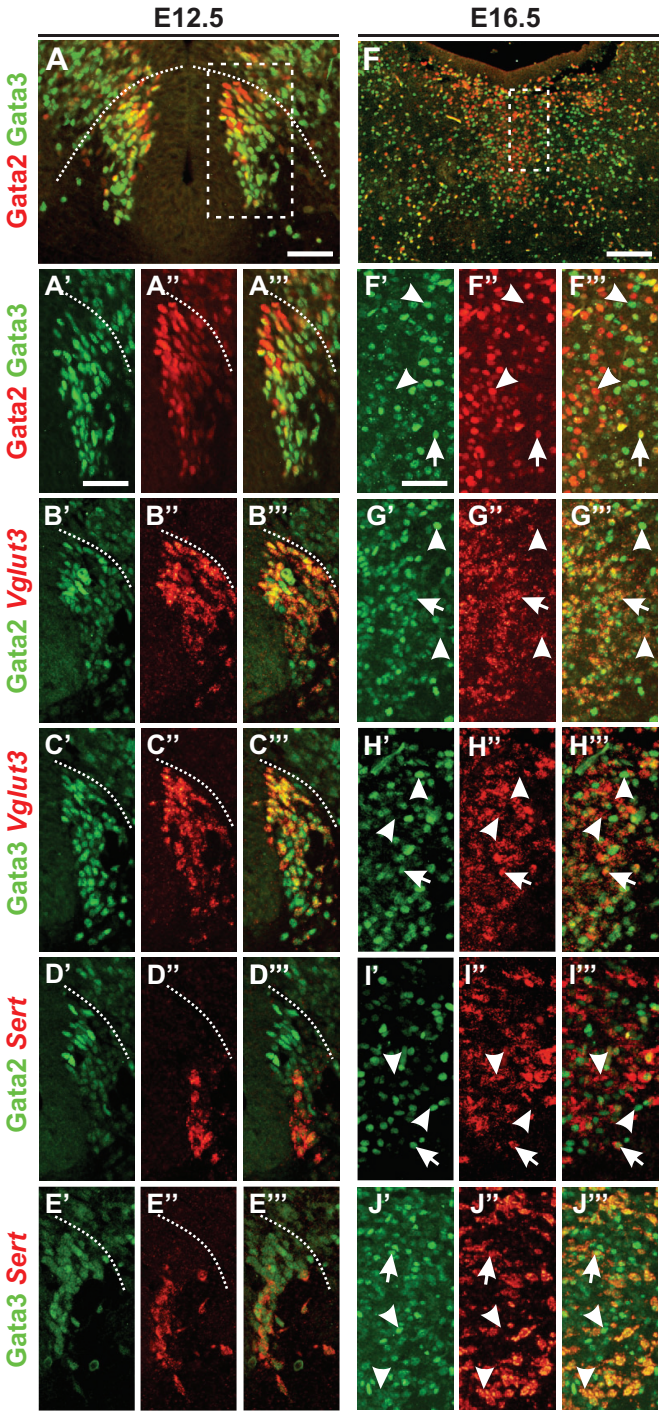

Fig. 4. Gata2 and Gata3 expression in *Vglut3*$^{+}$ and *Sert*$^{+}$ precursors. (A-A‴, F-F‴) Gata2 and Gata3 IHC at E12.5 and E16.5; boxed regions are shown at higher magnification in A′-A‴,F′-F‴. (B′-B‴,G′-G‴) Gata2 IHC and *Vglut3* ISH. (C′-C‴,H′-H‴) Gata3 IHC and *Vglut3* ISH. (D′-D‴,I′-I‴) Gata2 IHC and *Sert* ISH. (E′-E‴,J′-J‴) Gata3 IHC and *Sert* ISH. Arrows indicate co-expression and arrowheads single-positive cells. Dotted lines indicate the dorsal border of Nkx2-2 expression on a parallel section. Scale bars: 50 µm (A,A′,F′); 100 µm (F).

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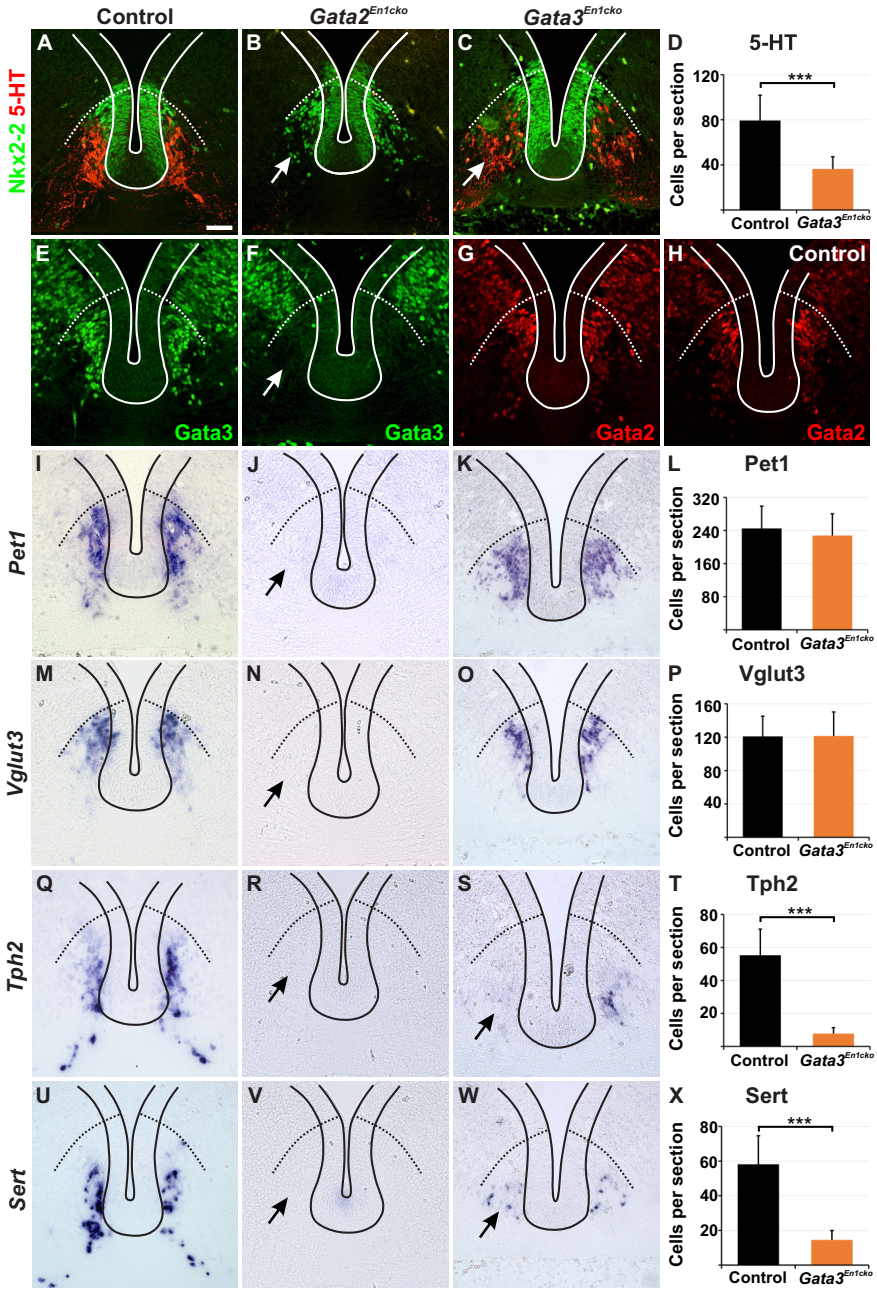

Fig. 5. Defective serotonergic neuron development in *Gata2*^{En1cko} and *Gata3*^{En1cko} at E12.5. (A-C) Nkx2-2 and 5-HT IHC. (E,F) Gata3 IHC. (G,H) Gata2 IHC. (I-K,M-O,Q-S,U-W) *Pet1*, *Vglut3*, *Tph2* and *Sert* ISH. (D,L,P,T,X) Quantification of 5-HT-, *Pet1*-, *Vglut3*-, *Tph2*- and *Sert*-positive cells in control and *Gata3*^{En1cko} embryos. Bars represent mean±s.d. ***P<0.001 (two-tailed Student's *t*-test). Arrows indicate defective activation of serotonergic markers in *Gata2*^{En1cko} and *Gata3*^{En1cko}. Dotted lines mark borders between domains rpvMN and rp3. Scale bar: 50 µm.

phenotype, as we detected no expression of 5-HT, Gata3, *Pet1*, *Tph2*, *Vglut3* or *Sert* in them (Fig. 5B,F,J,N,R,V). As the *En1*^{Cre} allele drives recombination throughout the midbrain and r1, we investigated whether a more specific inactivation of *Gata2* in the ventral r1 using the *Nkx2-2*^{Cre} allele also leads to defective serotonergic neuron development. Similar to the *Gata2*^{En1cko} mutants, the expression of 5-HT, Vglut3 and Sert was absent or greatly reduced in the *Nkx2-2*^{Cre};*Gata2*$^{flox/flox}$ embryos at E12.5 (Fig. S6A-H). Few remaining serotonergic neurons were detected at E18.5 (Fig. S6I-N). These remain probably because of incomplete/late recombination by the *Nkx2-2*^{Cre} (Fig. S6E,F,H, arrowheads). Thus, the failure in serotonergic neuron differentiation in the *Gata2*^{En1cko} embryos is likely to be due to an intrinsic Gata2 function in the precursors derived from the Nkx2-2^{+} progenitors.

Using cDNA microarrays, we further profiled gene expression changes between the wild-type and *Gata2*^{En1cko} basal r1 at E12.5 and identified additional gene products downregulated in the *Gata2*^{En1cko} (Table 1). Gene ontology analyses and comparisons with published serotonergic neuron transcriptomes (Okaty et al., 2015) revealed that the genes downregulated in the *Gata2*^{En1cko} embryos included many that were expressed in serotonergic neurons and important for their function (Table S3). We validated and complemented these results by *in situ* mRNA hybridisation (ISH) experiments. All serotonergic neuron-specific genes we analysed were downregulated in the ventral r1 of E12.5 *Gata2*^{En1cko} embryos (Table 1; data not shown). Some of the genes we analysed by ISH were also expressed in the r1 outside the serotonergic domain. This probably explains why they were not detected among the downregulated transcripts in the cDNA microarray profiling.

Taken together, our data support the hypothesis that Gata2 acts at the top of the gene regulatory hierarchy in the post-mitotic precursors of the ventral r1, leading to the differentiation and development of the serotonergic phenotype of both *Vglut3*- and *Sert*-expressing serotonergic neuron subtypes.

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Table 1. Genes downregulated in *Gata2*^{En1cko} and *Gata3*^{En1cko} at E12.5

	Microarray (*Gata2*^{En1cko} compared with wild type)		ISH (serotonergic neurons)	
Symbol	Fold change	Adjusted *P*-value*	*Gata2*^{En1cko}	*Gata3*^{En1cko}
Tph2	−15.597	1.175e−06	–	–
Cryba2	−8.243	1.015e−05	–	+
Sert	−7.29	1.175e−06	–	–
Vglut3	−6.52	7.963e−05	–	+
Gfpt1	−5.481	2.311e−07	n.a.	n.a.
Gchfr	−3.348	0.0004288	n.a.	n.a.
Scg2	−2.702	2.885e−05	–	n.a.
Chgb	−2.271	0.003697	n.a.	n.a.
Pet1	−1.96	0.002909	–	+
Nt5c3	−1.863	0.007053	n.a.	n.a.
Syt1	−1.815	0.007955	n.a.	n.a.
Csnk2a1	−1.761	0.005796	n.a.	n.a.
Mad (*Mxd1*)	−1.75	0.0004881	n.a.	n.a.
Adamts9	−1.709	0.003697	n.a.	n.a.
Gprin2	−1.667	0.004920	n.a.	n.a.
Ddc	−1.66	0.02700	–	+
Gch1	−1.583	0.02518	n.a.	n.a.
Akap9	−1.543	0.01969	n.a.	n.a.
C130021I20Rik	−1.54	0.02570	n.a.	n.a.
Ehmt1	−1.504	0.03558	n.a.	n.a.
Gata2	n.d.		–	+
Gata3	n.d.		–	–
Lmx1b	n.d.		–	+
Foxp1	n.d.		–	n.a.
Vmat2 (*Slc18a2*)	n.d.		–	+
Uncx	n.d.		–	n.a.

*Genes with *P*<0.05 shown.
–, lost/reduced expression; +, expression not changed; n.a., not analysed; n.d., not detected.

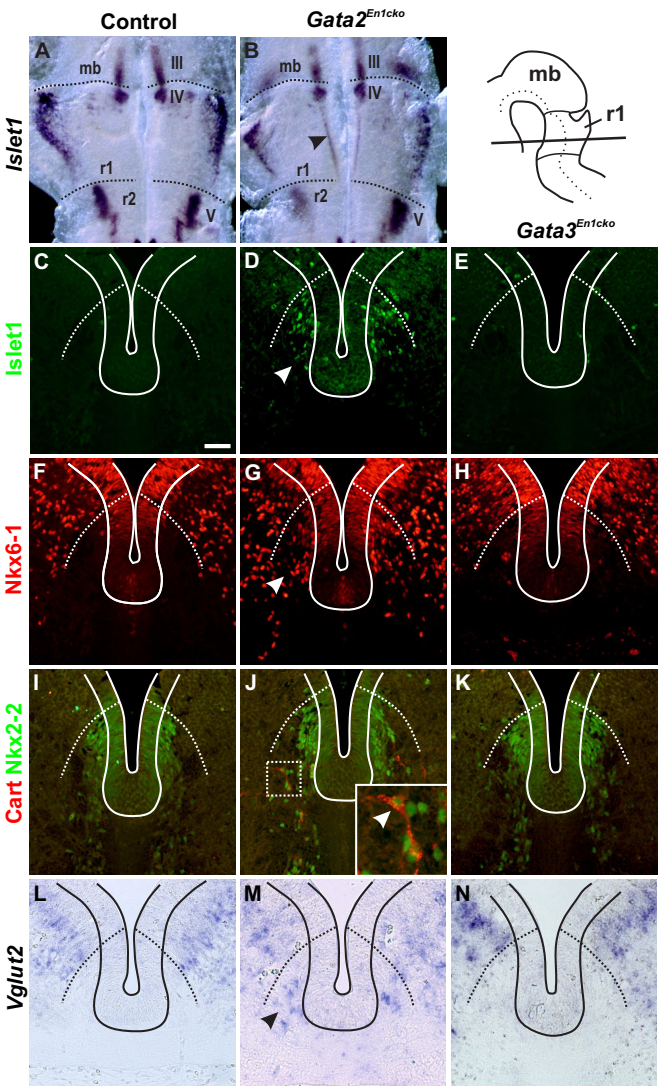

Fig. 6. Transformation of the serotonergic precursor identity in *Gata2*^{En1cko}. (A,B) *Islet1* whole-mount ISH at E12.5. Dotted lines indicate midbrain-r1 and r1-r2 borders. (C-E) Islet1 IHC. (F-H) Nkx6-1 IHC. (I-K) Cart and Nkx2-2 IHC. (L-N) *Vglut2* ISH. Arrowheads indicate ectopic expression in the *Gata2*^{En1cko} post-mitotic precursors. Dotted lines in C-N mark the rp3-rpvMN border. In J, boxed area is shown at higher magnification in the inset. Schematic indicates level of section shown in C-N. mb, midbrain; III oculomotor nucleus; IV, trochlear nucleus; V, trigeminal nucleus. Scale bar: 50 µm.

Gata2 directs r1 neuronal precursors to a serotonergic fate in preference to alternative fates

We then investigated whether the neuronal precursors in the ventral r1 had adopted a different neuronal phenotype in the absence of *Gata2* gene function. These phenotypes might correspond to the ones taken by neuronal precursors derived from the rp3, rpMN or other ventral r1 domains (Fig. 1). Indeed, concomitant with the loss of the serotonergic neuron markers, we found ectopic induction of *Islet1* (*Isl1*) and *Vglut2* (*Slc17a6*) expression in the ventral r1 of E12.5 *Gata2*^{En1cko} embryos (Fig. 6B,D,M). Moreover, the expression of Nkx6-1, which is mostly confined to the proliferative progenitors in the wild type, was maintained or re-activated in the post-mitotic precursors in the *Gata2*^{En1cko} embryos (Fig. 6G; Fig. S7E). Interestingly, the precursors derived from different Nkx2-2 subdomains adopted distinct phenotypes in the *Gata2*^{En1cko} mutants: we observed upregulation of Islet1 primarily in the dorsal precursors, but *Vglut2* in the ventral precursors (Fig. 6; data not shown).

Notably, despite the induction of Islet1, a motor neuron marker, we observed no ectopic expression of general cholinergic markers [ChAT, VAChT (Slc18a3)] or markers of trochlear (*Phox2b*, *Phox2a*), visceral (*Tbx20*) or somatic [HB9 (Mnx1)] motor neurons in *Gata2*^{En1cko} mutants at E12.5-E18.5 (data not shown). To gain further insight into the enigmatic phenotype adopted by the ventral r1 precursors in *Gata2*^{En1cko} embryos, we analysed genes for which expression was upregulated in the *Gata2*^{En1cko} embryos in our microarray-based gene-expression analyses (Table 2). One of these, cocaine and amphetamine-regulated transcript (*Cart*), encoding for a neuropeptide expressed in the centrally projecting preEdinger–Westphal nucleus located near the ventral midline of the midbrain (Kozicz et al., 2011), was indeed ectopically expressed in some cells of *Gata2*^{En1cko} ventral r1 at E12.5 (Fig. 6J). These Cart^{+} cells also expressed Nkx6-1 but most of them did not express Islet1 or *Vglut2* (Fig. S8A-C).

We then investigated whether the transformed neural precursors contributed to nuclei in the brain. Ectopic Islet1^{+} and Cart^{+} neurons were detected in the *Gata2*^{En1cko} ventral r1 at E13.5 and E14.5 (data not shown). However, we did not find increased numbers or ectopic Islet1^{+}, Nkx6-1^{+} or Cart^{+} neurons in the preEdinger–Westphal nucleus or in the r1 area at E18.5 (Fig. S8D-K; data not shown). The fate of ectopic *Vglut2*$^{+}$ cells remains unclear as *Vglut2* is widely expressed in the ventral r1 at later stages of development and other population-specific markers are lacking. Aside from Islet1, Nkx6-1, *Vglut2* and Cart, we observed no upregulation of dopaminergic (Th)

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Table 2. Genes upregulated in *Gata2*^{En1cko} at E12.5

	Microarray (*Gata2*^{En1cko} compared with wild type)	
Symbol	Fold change	Adjusted *P*-value*
Pkd2l1	1.616	0.0043
Acta2	1.558	0.018
Metrn	1.631	0.022
Cart	1.676	0.036

*Genes with $P<0.05$ shown.

or GABAergic (*Gad1*) neuron markers, nor ectopic expression of *Sim1*, a gene expressed in the analogous ventral V3 domain of the spinal cord, in the *Gata2*^{En1cko} r1. The expression of Olig2 was also still restricted to cells adjacent to the Nkx2-9^{+} region (Fig. S9).

Thus, in the absence of Gata2, the postmitotic precursors produced in the ventral r1 might switch their phenotype from serotonergic to other neuronal identities. These new identities remain largely unclear, but include precursors transiently expressing Islet1, precursors resembling the neurons found in the nearby preEdinger–Westphal nucleus, as well as precursors of *Vglut2*$^{+}$ glutamatergic neurons.

Distinct requirements for Gata2 and Gata3 in subpopulations of serotonergic precursors

Downstream of Gata2, Gata3 may belong to the general transcriptional regulatory cascade leading to full activation of serotonergic neuron gene battery. However, these two Gata factors also showed differences in their relative expression in the serotonergic neuron subgroups (see above). To understand better the specific functions of Gata3 in serotonergic neuron development in the r1, we analysed *Gata3*^{En1cko} embryos and compared them with the Gata2 loss-of-function phenotype.

In contrast to the *Gata2*^{En1cko} mutants and consistent with earlier reports (Pattyn et al., 2004), we observed 5-HT-positive serotonergic precursors in *Gata3*^{En1cko} embryos at E12.5, albeit in severely reduced numbers compared with the wild type (Fig. 5C,D). Expression analyses of genes characteristic for serotonergic neurons suggested that Gata3 is required for the expression of only a subset of these markers, as we observed a noticeable downregulation of *Tph2* and *Sert*, but not Gata2, *Pet1* or *Vglut3* in the *Gata3*^{En1cko} embryos (Fig. 5G,K,L,O,P,S,T,W,X; Table 1). In contrast to the *Gata2*^{En1cko}, we did not observe upregulation of Islet1, Nkx6-1, Cart or *Vglut2* in *Gata3*^{En1cko} ventral r1 precursors (Fig. 6E,H,K,N).

We then investigated how the developmental defects impact the cell type distribution in the dorsal and median raphe complexes in *Gata2*^{En1cko} and *Gata3*^{En1cko} brains. Consistent with the phenotypes of *Gata2*^{En1cko} embryos at E12.5, we detected loss of most serotonergic markers, including 5-HT, *Tph2* and *Sert* in *Gata2*^{En1cko} mutants at E18.5 (Fig. 7D,E,M,N; data not shown). By including a Cre-recombinase-based midbrain-r1 cell lineage reporter *ROSA26*$^{mT\text{-}mG}$ (*En1*$^{Cre/+}$;*Gata2*$^{flox/flox}$;*ROSA26*$^{mT\text{-}mG/+}$) we showed that the loss of the serotonergic neuron markers in the *Gata2*^{En1cko} mutants was complete and specific to the dorsal and median raphe serotonergic neuron populations (Fig. S10A,B), earlier shown to be derived from the r1 (Jensen et al., 2008). In E18.5 *Gata3*^{En1cko} mutants, again reflecting their E12.5 phenotype, we detected decreased numbers of 5-HT-, *Sert*- and *Tph2*-positive serotonergic neurons, including both *Sert*$^{+}$ and *Sert*$^{+}$*Vglut3*$^{+}$ subtypes in different regions of the dorsal raphe (Fig. 8A-F,J-M; Fig. S11). Interestingly, despite the loss of *Tph2* and *Sert* expression, we still detected abundant *Vglut3* expression in the central part of the dorsal raphe (Fig. 8G-K; Fig. S11).

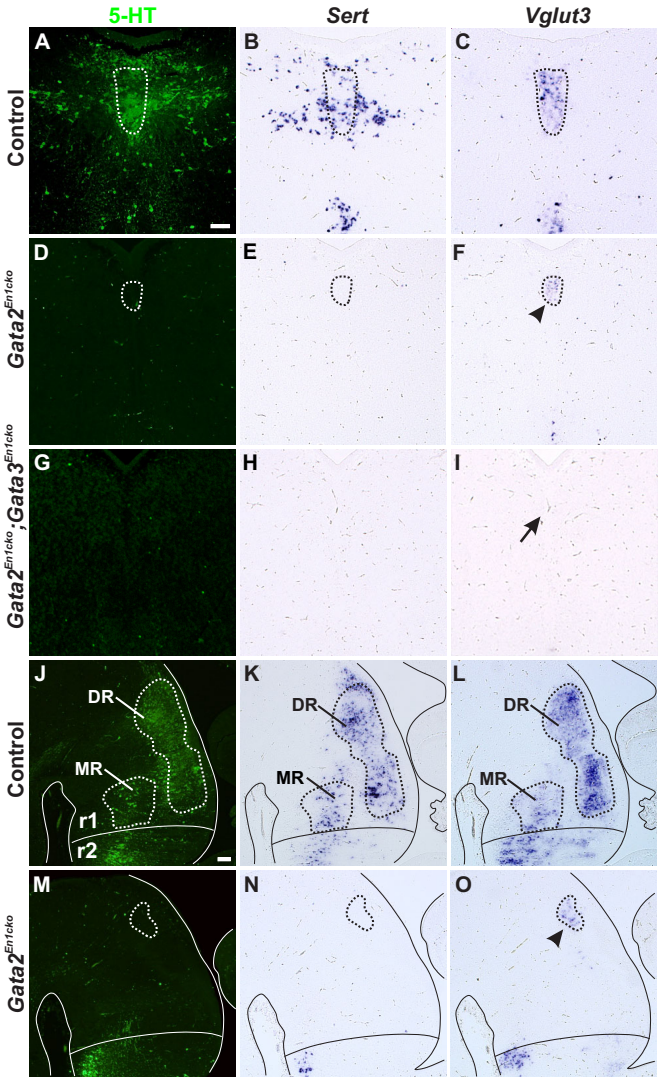

Fig. 7. Development of dorsal and median raphe neurons in *Gata2*^{En1cko} and *Gata2*^{En1cko}; *Gata3*^{En1cko}. Coronal (A-I; see Fig. S2P for the sectioning plane) and sagittal (J-O) sections of E18.5 embryos. (A,D,G,J,M) 5-HT IHC. (B,E,H,K,N) *Sert* ISH. (C,F,I,L,O) *Vglut3* ISH. Arrowheads in F,O indicate *Vglut3*$^{+}$ cells in *Gata2*^{En1cko}. Arrow in I shows loss of *Vglut3*$^{+}$ cells in *Gata2*^{En1cko}; *Gata3*^{En1cko}. Dotted lines indicate *Vglut3* expression area on parallel sections. The r1-r2 boundary is indicated by a horizontal line in J-O. DR, dorsal raphe; MR, median raphe. Scale bars: 100 µm.

Thus, our loss-of-function analyses demonstrated differential requirements for Gata2 and Gata3 for the activation of genes defining serotonergic neuron identity. These differences also correlated with the expression levels of Gata2 and Gata3 in the *Vglut3*$^{+}$ and *Sert*$^{+}$ serotonergic neuron subgroups early in the development.

Gata2 and Gata3 cooperatively regulate the development of the r1-derived dorsal raphe glutamatergic neurons

Previous studies have shown that the dorsal raphe contains both serotonergic and non-serotonergic Vglut3^{+} cells. Our analyses showed that the expression of *Vglut3* was largely abolished in the *Gata2*^{En1cko} mutants, but it seemed unaffected in *Gata3*^{En1cko} embryos (Fig. 7F,O; Fig. 8H,I). Interestingly, however, we detected a spared *Vglut3*-expressing neuronal population in a central region of the dorsal raphe in the *Gata2*^{En1cko} mutants (Fig. 7F,O). These *Vglut3*$^{+}$ neurons were devoid of 5-HT and thus might correspond to the recently identified

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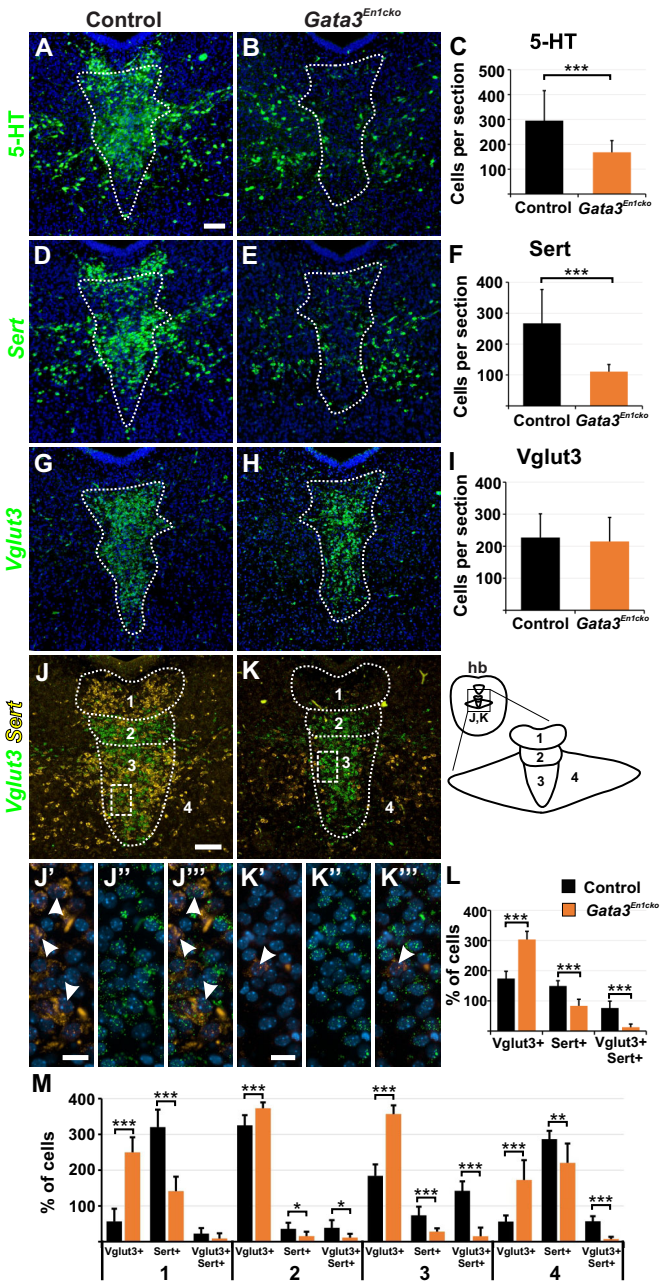

Fig. 8. Development of dorsal and median raphe neuron subgroups in *Gata3*^{En1cko}. (A-K‴) Coronal sections of E18.5 embryos. (A,B) 5-HT IHC. (D,E) *Sert* ISH. (G,H) *Vglut3* ISH. (J,K) Combined *Vglut3* and *Sert* ISH; boxed areas are shown at higher magnification in J′-J‴,K′-K‴. Arrowheads mark Sert$^+$ cells. (C,F,I) Quantification of 5-HT$^+$, *Sert*$^+$ and *Vglut3*$^+$ cells in control and *Gata3*^{En1cko} embryos. (L,M) Relative amounts of *Sert*$^+$, *Vglut3*$^+$ and *Vglut3*$^+$*Sert*$^+$ cells in the whole dorsal raphe (L) and in four regions of dorsal raphe (M) at E18.5. Regions are indicated in the schematic and in J,K. Bars represent mean±s.d. **P*<0.05, ***P*<0.01, ****P*<0.001 (two-tailed Student's *t*-test). Dotted lines in A,B,D,E,G,H indicate *Vglut3* expression area on parallel sections. hb, hindbrain. Scale bars: 20 µm (J′,K′); 100 µm (A).

Vglut3$^+$ dorsal raphe glutamatergic neuron subgroup projecting to the VTA (Hioki et al., 2010; Liu et al., 2014; McDevitt et al., 2014; Qi et al., 2014). The spared *Vglut3*$^+$ cells first appeared in the ventral r1 of the *Gata2*^{En1cko} embryos at E14.5 (data not shown) and may thus represent a relatively late-born subgroup. To investigate when the *Vglut3*$^+$ glutamatergic neurons are born, we administered 5-ethynyl-2′-deoxyuridine (EdU) to pregnant females at E10.5, E11.5, E12.5 or E13.5, and analysed the dorsal raphe of wild-type and *Gata2*^{En1cko} embryos at E18.5. The majority of the 5-HT$^+$ and *Vglut3*$^+$ neurons in the dorsal raphe of wild-type embryos were labelled when EdU was given at E10.5, whereas only few of the 5-HT$^+$ cells were labelled when EdU was given at the later stages (Fig. 9B,D,G; Fig. S12B,D; data not shown), consistent with earlier reports (Jacob et al., 2007). In contrast to the 5-HT$^+$ neurons, some *Vglut3*$^+$ glutamatergic neurons in the central dorsal raphe of both wild-type and *Gata2*^{En1cko} embryos were labelled when EdU was administered at E11.5, E12.5, but not any when given at E13.5 (Fig. 9A,C,E-G; Fig. S12A,C; data not shown). This suggests that, in the dorsal raphe, the *Vglut3*$^+$ glutamatergic neurons are born later than serotonergic neurons, and many of them exit the cell cycle only after E12.5 (Fig. 9G).

Next, using Cre-recombinase-based fate mapping, we studied the developmental origin of the *Vglut3*$^+$ glutamatergic neurons. The whole dorsal raphe area was efficiently labelled in E18.5 *En1*$^{Cre/+}$;*ROSA26*$^{TdTomato/+}$ brains, suggesting that the *Vglut3*$^+$ population is not derived from posterior rhombomeres (Fig. S10C-E). To gain insights into the origin of the *Vglut3*$^+$ glutamatergic neurons within the r1 and their lineage relationship with the dorsal raphe serotonergic neurons, we analysed the pattern of Cre-mediated recombination in the dorsal raphe of E18.5 *Nkx2-2*$^{Cre/+}$;*ROSA26*$^{TdTomato/+}$ embryos. In addition to the dorsal raphe serotonergic neurons, the centrally located *Vglut3*$^+$ population was also efficiently labelled (Fig. 10A-C). Interestingly, although these *Vglut3*$^+$ cells were negative for the serotonergic marker 5-HT, they still expressed *Pet1* (Fig. 10D-F). However, the level of *Pet1* expression was lower in the *Vglut3*$^+$ glutamatergic neurons compared with the adjacent serotonergic cells (Fig. 10D,F,G). These results suggest that, similar to the serotonergic neurons, the *Vglut3*$^+$ glutamatergic neurons in the central dorsal raphe are derived from *Nkx2-2*$^+$ progenitors in the ventral r1.

Finally, as the dorsal raphe glutamatergic neurons share lineage with the r1-derived serotonergic neurons, and yet were spared in both *Gata2*^{En1cko} and *Gata3*^{En1cko} brains, we addressed the possibility of redundancy between the Gata TFs for the development of these cells. Supporting this, we detected no *Vglut3* expression in *Gata2*^{En1cko};*Gata3*^{En1cko} double mutants (*En1*$^{Cre/+}$;*Gata2*$^{flox/flox}$;*Gata3*$^{flox/flox}$) (Fig. 7I). Altogether, our results show that differentiation of both serotonergic and glutamatergic neurons of the dorsal raphe is regulated by Gata2 and Gata3, but relative differences in the requirements for these factors may delineate distinct neuronal subgroups.

DISCUSSION

Considering the multifaceted functions of the serotonergic system, a substantial level of heterogeneity can be expected in its cellular composition. Knowledge of the mechanisms guiding serotonergic neuron differentiation should lead to a better understanding of the serotonergic cell types. We mapped the neural progenitor domains and their derivatives in the ventral r1, a region producing dorsal and median raphe serotonergic neurons. We studied how the cell types in the dorsal raphe are affected by the loss of function of the TFs Gata2 and Gata3, revealing an early heterogeneity among serotonergic neuron precursors, both in their molecular composition and in developmental regulatory mechanisms. We also provide information on the development of the dorsal raphe glutamatergic neurons, recently associated with brain reward circuitries.

Molecular diversity within the Nkx2-2-expressing neural progenitors in the ventral r1

Serotonergic neurons are generated from a narrow Nkx2-2$^+$ ventral neuroepithelial domain (Briscoe et al., 1999), yet Nkx2-2 is required for serotonergic neuron development only in the posterior hindbrain

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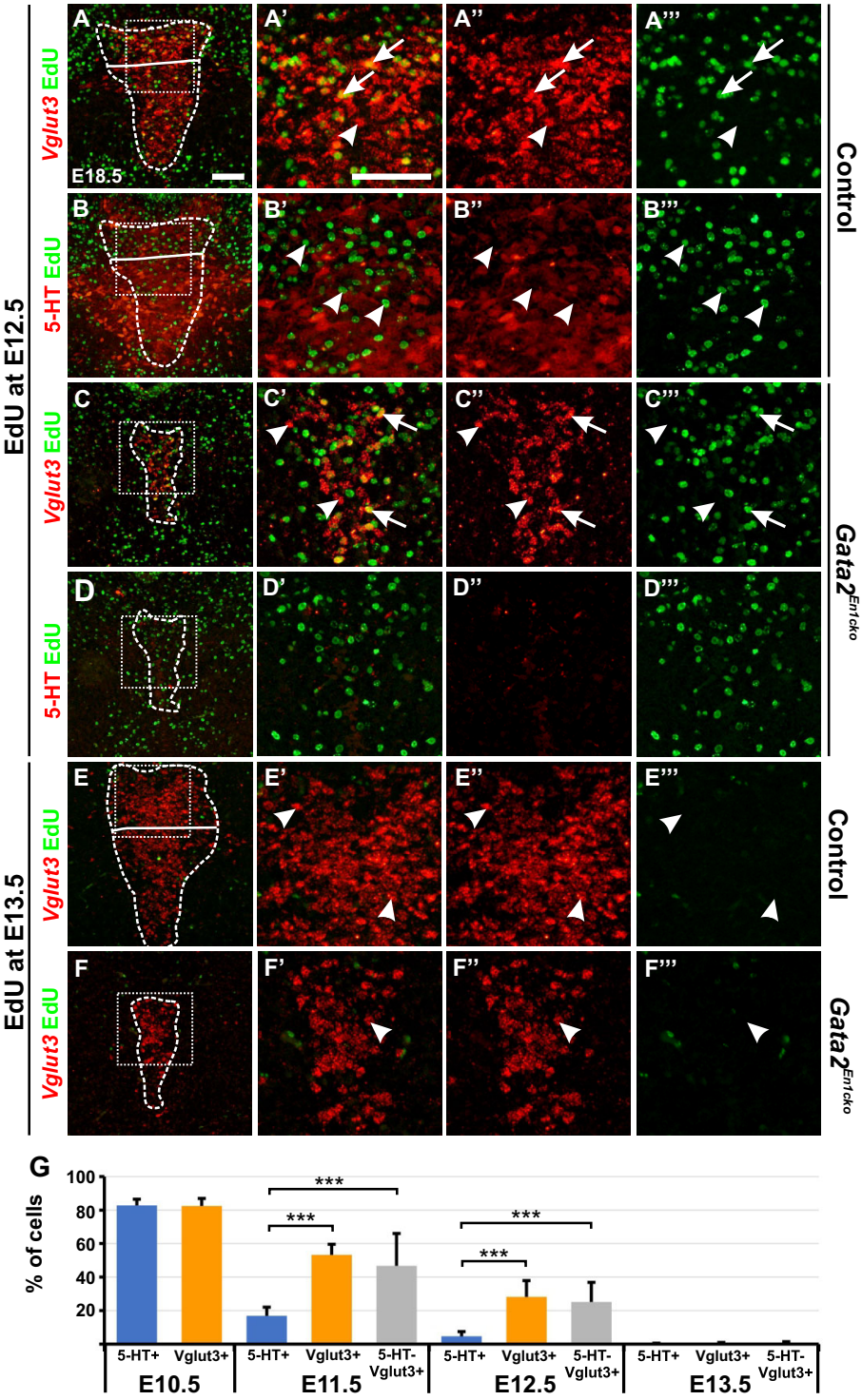

Fig. 9. Birth-dating of serotonergic and *Vglut3*$^{+}$ glutamatergic neurons in the dorsal raphe. (A-F‴) *Vglut3* ISH with EdU (A-A‴,C-C‴,E-F‴) and 5-HT IHC with EdU (B-B‴,D-D‴) in the dorsal raphe of E18.5 control and *Gata2*^{En1cko} embryos labelled with EdU at E12.5 or E13.5; boxed areas shown at higher magnification in A′-F‴. Dashed lines indicate the area of *Vglut3* expression, continuous lines mark the border of the region containing cells expressing *Vglut3* but not 5-HT. Arrows indicate cells that are labelled with EdU and express 5-HT or *Vglut3*. Arrowheads mark EdU-negative cells. (G) Proportion of 5-HT^{+}, *Vglut3*$^{+}$ and 5-HT^{-}*Vglut3*$^{+}$ cells in the dorsal raphe, when EdU was given E10.5, E11.5, E12.5 and E13.5. Bars represent mean±s.d. ***P<0.001 (two-tailed Student's t-test). Scale bars: 100 µm.

and not in the r1. A possible explanation for this is functional redundancy of Nkx2-2 with a related TF, Nkx2-9 (Deneris and Wyler, 2012). However, although our results confirm that Nkx2-9 is co-expressed with Nkx2-2 in the ventral r1, Nkx2-9 expression does not coincide with the region producing serotonergic neurons. Rather, Nkx2-9 marks a more dorsal domain adjacent to newly born oligodendrocytes. Thus, the Nkx2-2-expressing neuroepithelium could be subdivided into a dorsal domain producing oligodendrocytes (rpvMN) and a ventral domain producing serotonergic neurons (rp3) (Fig. 11A). We also show that two other homeodomain TFs, Nkx6-1 and Nkx6-2 are expressed in the mouse rp3, consistent with earlier reports of Nkx6-1 expression in chicken serotonergic progenitors (Craven et al., 2004). In the mouse rp3, both Nkx6-1 and Nkx6-2 are expressed in a dorsoventral gradient. These observations reveal molecular heterogeneity among rp3 progenitors and suggest that they may be further divided into distinct subgroups.

Gata2 as a serotonergic neuron terminal selector TF

Following neuroepithelial patterning, neuron type-specific gene expression is initiated by terminal selector TFs that are expressed

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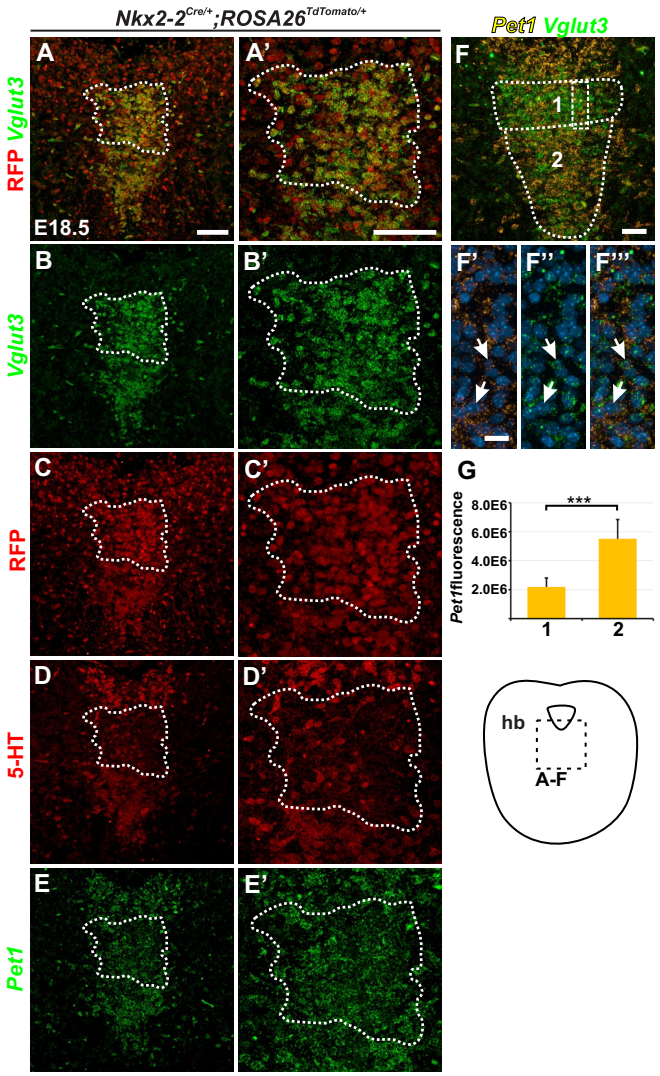
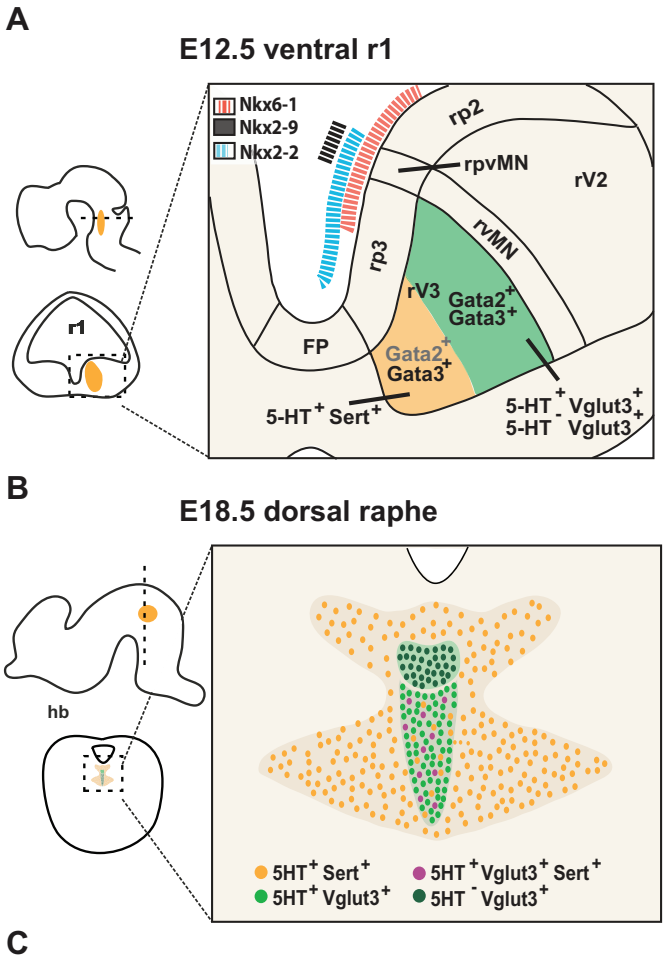


Fig. 10. Developmental origins of *Vglut3*$^{+}$ dorsal raphe glutamatergic neurons. (A-E′) Coronal sections of the dorsal raphe of E18.5 *Nkx2-2*$^{Cre/+}$;*ROSA26*$^{TdTomato/+}$ embryo. (A-C′) *Vglut3* ISH with RFP IHC; boxed areas shown at higher magnification in A′-C′. (D,D′) 5-HT IHC on an adjacent section; boxed area shown at higher magnification in D′. (E,E′) *Pet1* fluorescent ISH; boxed area shown at higher magnification in E′. Dotted line marks *Vglut3*$^{+}$5-HT^{-} area. (F) Combined *Pet1* and *Vglut3* ISH in E18.5 wild-type dorsal raphe; boxed areas shown at higher magnification in F′-F‴. Arrows indicate double-positive cells. (G) Quantification of the *Pet1* ISH signal in the *Vglut3*$^{+}$5-HT^{-} (1) and *Vglut3*$^{+}$5-HT^{+} (2) regions (identified on parallel sections, indicated in F). Bars represent mean±s.d. ***P<0.001 (two-tailed Student's t-test). Schematic indicates the area shown in A-F. hb, hindbrain. Scale bars: 50 µm (F); 100 µm (A,A′).



C

Cell type:	*Gata2*^{En1cko}	*Gata3*^{En1cko}	*Gata2*^{En1cko};*Gata3*^{En1cko}
5HT^{+} Sert^{+}	Lost	Decreased	Lost
5HT^{+} Vglut3^{+}	Lost	Decreased	Lost
5HT^{+}Vglut3^{+} Sert^{+}	Lost	Decreased	Lost
5HT^{-}Vglut3^{+}	Present	Increased	Lost

Fig. 11. Model of development and neuronal diversity in the dorsal raphe. (A) Development of different cell types from the Nkx2-2-positive progenitor domain in the embryonic ventral r1. Coloured bars mark the expression of Nkx2-2, Nkx2-9 and Nkx6-1 in the ventricular zone progenitors, which give rise to precursors with differences in the expression of Gata2/3 selector TFs and neuronal subtype markers. (B,C) Neuronal diversity in the dorsal raphe at E18.5 (B) and dependency of different neuron subgroups on Gata2 and Gata3 (C). FP, floor plate; hb, hindbrain; rV2,3, rhombencephalic V2, V3 precursor domain.

upon cell cycle exit (Hobert, 2011). Pet1 has been suggested to operate as a terminal selector of the serotonergic neurons as it is thought to be specific for the serotonergic lineage and directly regulates genes belonging to the serotonergic gene battery. However, Pet1 is not absolutely required for serotonergic neuron development and Pet1-deficient neural precursors are not known to be redirected to an alternative fate (Deneris and Wyler, 2012).

Our results suggest that Gata2 fulfils many criteria for a terminal selector during early neuronal differentiation of serotonergic neurons. Gata2 is activated upon cell cycle exit and without Gata2 function there is a complete loss of serotonergic identity and appearance of cells with alternative phenotypes. In addition, we show that Gata2 is required for the activation of the closely related TF Gata3 in the serotonergic precursors. This is in contrast to the commonly held view of independent activation of *Gata2* and *Gata3* during serotonergic differentiation (Deneris and Wyler, 2012), which is apparently due to the expression of *Gata3* in the neighbouring GABAergic precursors (Kala et al., 2009; Achim et al., 2012). The expression of *Gata3* continues in the adult serotonergic neurons and Gata3 contributes to the maintenance of some of the serotonergic neuron-specific genes (Liu et al., 2010). Thus, the serotonergic terminal selector function may be viewed as a sequential and joint activity of a TF network including Gata2, Gata3, Lmx1b and Pet1.

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The combinatorial code for postmitotic neuronal fate selection is also suggested by the context dependence of Gata2 function. When overexpressed, Gata2 can induce serotonergic fate only in the r1 (Craven et al., 2004). However, Gata2 can operate as a selector of other neuronal identities depending on the brain region (Kala et al., 2009; Virolainen et al., 2012). These separate functions could be, at least partly, due to distinct Gata co-factors expressed in postmitotic GABAergic and serotonergic neuron precursors (Lahti et al., 2016). The ectopic neurons observed in the ventral r1 of the *Gata2*^{En1cko} mutants could reflect neurons also produced in the wild-type r1, but at an earlier time point in development. However, their exact identity remains unknown.

Generation of neuronal diversity in the dorsal raphe

One of the differences between the serotonergic neurons is the expression of co-neurotransmitters, such as glutamate. We show that in the dorsal raphe, most of the medial serotonergic neurons are *Vglut3*$^{+}$ and some of these also express *Sert* (see below), whereas the lateral neurons do not express *Vglut3* but are *Sert*$^{+}$. This is in contrast to earlier studies, in which *Vglut3* and *Sert* expression was reported to largely colocalise (Gras et al., 2002), although a later study showed that Vglut3 and Sert are detected in distinct nerve terminals in the forebrain (Amilhon et al., 2010).

Our results suggest that the early precursors for *Vglut3*$^{+}$ and *Sert*$^{+}$ serotonergic neurons are spatially distinct and can be characterised by different expression and function of Gata2 and Gata3. During neurogenesis, both dorsal *Vglut3*$^{+}$ and ventral *Sert*$^{+}$ precursors require Gata2, whereas Gata3 is needed for differentiation of ventral Sert^{+} precursors. However, in addition to differentiation of the *Sert*$^{+}$ neurons, Gata3 appears to be important for the full serotonergic identity of the *Vglut3*$^{+}$ neurons (see below). Thus, distinct serotonergic precursor populations diverge soon after cell cycle exit and show differences in their requirements for terminal selector TFs (Fig. 11A). Our results suggest that the *Vglut3*$^{+}$ cells produced from the more dorsal Nkx2-2^{+} domain finally assume a central position in the dorsal raphe complex. Similar dorsolateral-to-medial rearrangement has been reported during development of the midbrain dopaminergic complex (Panman et al., 2014).

Later, neurons expressing both *Vglut3* and *Sert* were observed in the central part of the dorsal raphe. Currently, it is difficult to establish whether these neurons are derived from *Vglut3* or *Sert* single-positive precursors. Similar to our observations, a recent expression profiling of serotonergic neuron subtypes revealed r2-derived populations of *Vglut3*^{low}, *Tph2/Sert*^{high} and *Vglut3*^{high}, *Tph2/Sert*^{low} cells in the median raphe, although this anti-correlation between *Vglut3* and *Tph2* was not detected in the r1-derived neurons (Okaty et al., 2015).

In addition to the serotonergic neurons, the dorsal raphe contains non-serotonergic *Vglut3*$^{+}$ glutamatergic neurons in its central subregion ('shell region' of the dorsal part) (Hioki et al., 2010) (Fig. 11B). Our results show that these *Vglut3*$^{+}$ glutamatergic neurons are developmentally related to the serotonergic neurons, as both of them have their origin in the *Nkx2-2*$^{+}$ progenitors and differentiation of both of these neuronal populations is dependent on Gata factors. Based on their EdU labelling and late appearance in the *Gata2*^{En1cko} mutants, the *Vglut3*$^{+}$ glutamatergic neurons appear to exit the cell cycle later than the serotonergic neurons. *Vglut3*$^{+}$ glutamatergic neurons also express *Pet1*, further supporting their developmental relationship with the serotonergic neurons. The precursors for *Vglut3*$^{+}$ serotonergic and glutamatergic neurons also show differences: the *Vglut3*$^{+}$ glutamatergic neurons express *Pet1* at a lower level and are completely lost only when both *Gata2* and *Gata3* are inactivated (Fig. 11C). Interestingly, in the *Gata3*^{En1cko} single mutants, the dorsal raphe serotonergic neurons assume a molecular phenotype reminiscent of the *Vglut3*$^{+}$ glutamatergic neurons, suggesting that the level/composition of the Gata selector complex distinguishes the fate of these neuronal subtypes. Further testing of this hypothesis would require unique markers for these neurons.

Conclusions and perspective

Our results suggest that a major choice in the serotonergic neuron subtype identity is established at the birth of these neurons. It is likely that this heterogeneity stems from differences in the regional characteristics of the serotonergic neuron progenitors modulating the activation and activity of the terminal selector TFs. The subtypes of serotonergic neurons are interesting from the perspective of behaviour and psychiatric disease. *Vglut3*-expressing serotonergic and glutamatergic neurons innervate dopaminergic neurons in the VTA and are important for reward signalling (Liu et al., 2014; McDevitt et al., 2014; Qi et al., 2014). Sert is the target of selective serotonin reuptake inhibitors (SSRIs), the commonly used anti-depressants. Unlike drugs affecting the dopaminergic pathways, SSRIs do not cause dependence. This might be partly due to *Sert*- and *Vglut3*-expressing neurons being largely distinct neuronal subgroups with unique functions.

MATERIALS AND METHODS

Mice

En1^{Cre} (Kimmel et al., 2000), *Gata2*^{flox} (Haugas et al., 2010), *Gata3*^{flox} (Grote et al., 2008), *ROSA26*$^{mT\text{-}mG}$ (Muzumdar et al., 2007), *ROSA26*^{TdTomato} (Madisen et al., 2010) and *Nkx2-2*^{Cre} (Balderes et al., 2013) alleles were on an outbred (ICR) background. Embryos were embedded in paraffin and sectioned at 5 µm. All analyses were confirmed using at least three biological replicates. Experiments were approved by the Laboratory Animal Centre, University of Helsinki, and the National Animal Experiment Board in Finland.

In situ mRNA hybridisation and immunohistochemistry

For *in situ* mRNA hybridisation (ISH), digoxigenin (DIG)-labelled antisense cRNA probes were used. For combined ISH and immunohistochemistry (IHC), the TSA Fluorescence Palette System (PerkinElmer) was used to visualise ISH signal and additional primary antibodies were added. For double ISH, DIG and fluorescein-labelled probes were used. Antibodies are listed in Table S1 and mRNA ISH probes in Table S2.

EdU labelling

ICR and *Gata2*$^{flox/flox}$ females mated with *En1*$^{Cre/+}$;*Gata2*^{flox} males were injected with EdU (10 mg/kg) at E10.5, E11.5, E12.5 or E13.5. EdU injections were carried out six times at 2 h intervals starting at noon and embryos were analysed at E18.5. EdU^{+} nuclei were visualised using the Click-iT EdU Alexa Fluor 488 or 555 Imaging Kit (Thermo Fisher Scientific).

mRNA expression profiling by cDNA microarray

Ventral r1 containing the whole basal plate region was dissected from E12.5 wild-type and *Gata2*^{En1cko} embryos. For both genotypes, three groups were generated, each consisting of six tissue samples. Total RNA was extracted with TriZol reagent and used for probe labelling. Illumina BeadChip (Mouse WG-6 2.0) microarrays were hybridised according to the manufacturer's protocol. The data set was normalised using the quantile normalisation method. Statistical testing was performed using the limma package from R and Bioconductor statistical analysis software.

Microscopy and statistical analysis

Images were taken with an Olympus AX70 microscope with Olympus DP70 camera, a Zeiss Axio Imager.M2 microscope with AxioCam HRc camera or

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Zeiss Axio Imager.M2 with Hamamatsu Orca-Flash 4.0 V2 camera. For quantification, positive cells were counted manually from sections (at least eight sections per embryo). ImageJ 1.50i was used to measure the *Pet1* ISH fluorescence (McCloy et al., 2014). Two-tailed Student's *t*-test was used for statistical analysis (*P<0.05, **P<0.01, ***P<0.001).

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Competing interests

The authors declare no competing or financial interests.

Author contributions

J.P. and M.S. conceived and supervised the project. M.H. and L.T. designed and performed experiments and analyzed data. K.A. carried out the microarray experiments. All the authors contributed to writing of the manuscript.

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Data availability

Microarray data have been deposited in NCBI's Gene Expression Omnibus under accession number GSE89354 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE89354).

Supplementary information

Supplementary information available online at
http://dev.biologists.org/lookup/doi/10.1242/dev.136614.supplemental

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Supplementary material

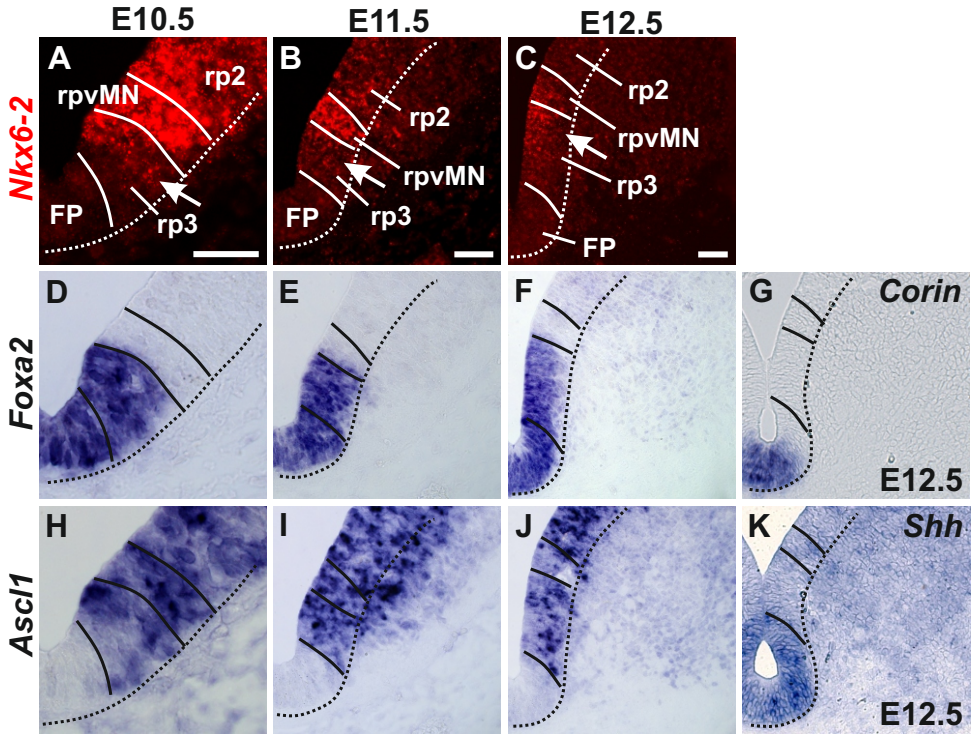

Figure S1. TFs expressed in the ventral r1 progenitors.

(A-K) ISH analysis of *Nkx6-2*, *Foxa2*, *Corin*, *Ascl1* and *Shh* expression on adjacent sections of E10.5-E12.5 wild-type embryos. The Nkx2-2 expressing subdomains rp3 and rpMN are indicated. Arrows point to graded expression of *Nkx6-2* in the rp3. FP, floor plate. Scale bars: 50 µm.

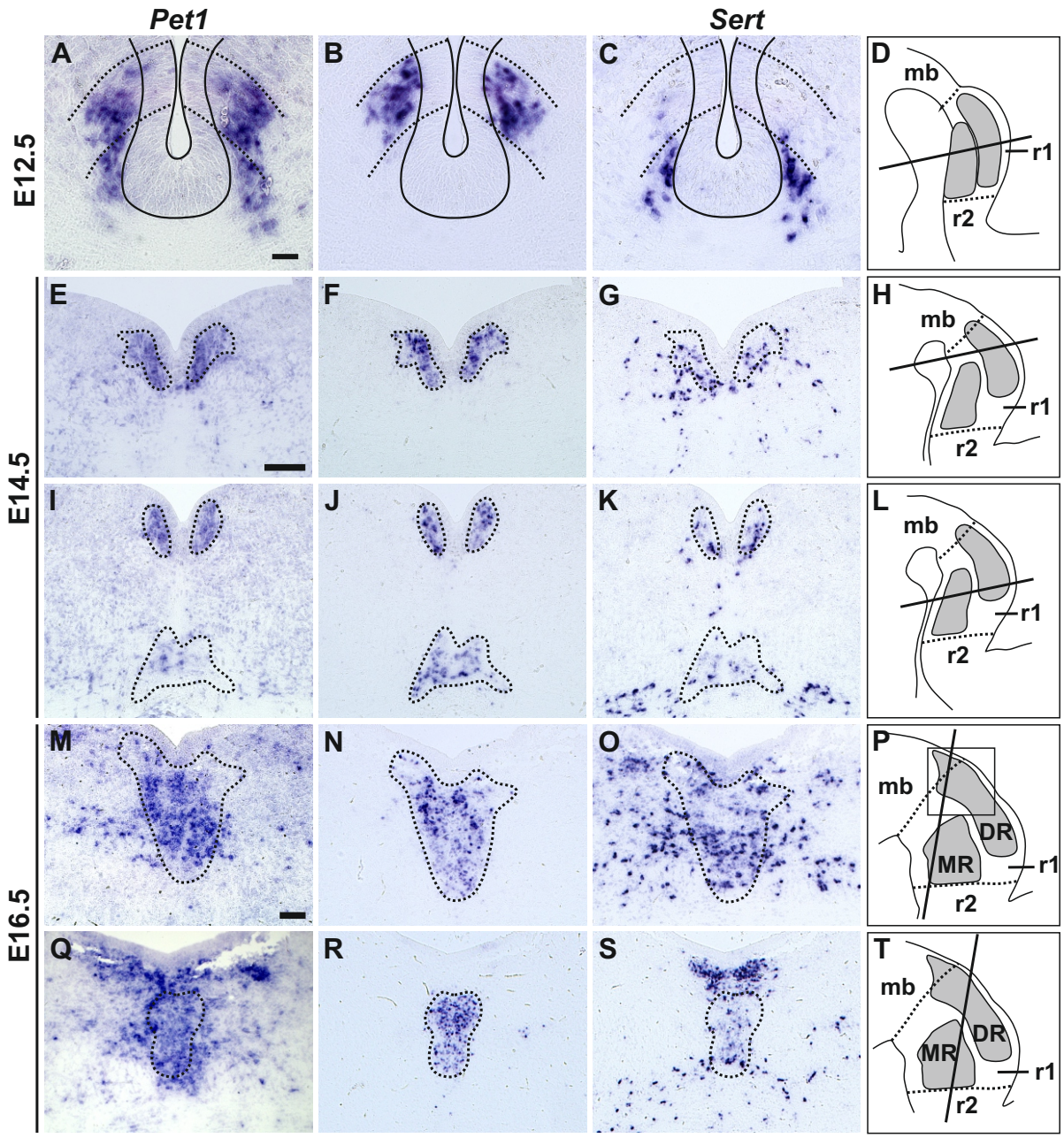

Figure S2. Development of Vglut3^{+} and Sert^{+} serotonergic neuron lineages.

Pet1, *Vglut3* and *Sert* ISH on adjacent sections at E12.5 (A-C), E14.5 (E-G,I-K) and E16.5 (M-O,Q-S), dotted lines mark expression area of *Vglut3*. The sectioning planes are indicated in (D,H,L,P,T), where grey colour marks the serotonergic populations. DR, dorsal raphe; MR, median raphe; mb, midbrain; r1, rhombomere 1; r2, rhombomere 2. Scale bars: 50 µm (A,E), 100 µm (M).

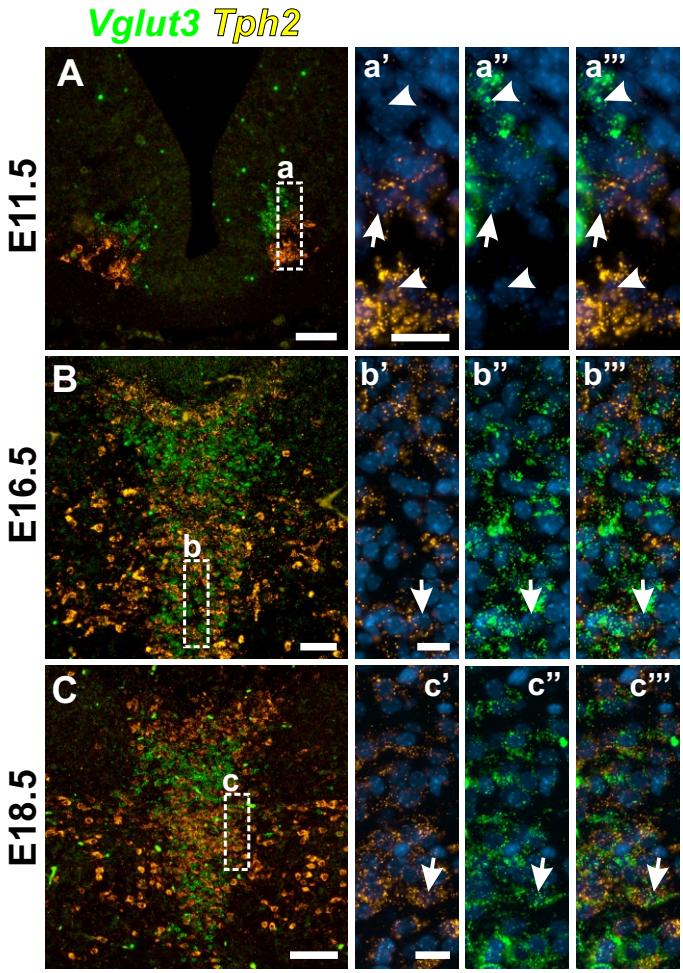

Figure S3. Vglut3^{+} and Tph2^{+} cells in the developing dorsal raphe.

(A-C) Combined *Vglut3* and *Tph2* ISH at E11.5, E16.5 and E18.5, close-ups in (a'-c'''). Arrows indicate double positive cells, arrowheads point to single positive cells. Scale bars: 20 µm (a'-c'), 50 µm (A,B), 100 µm (C).

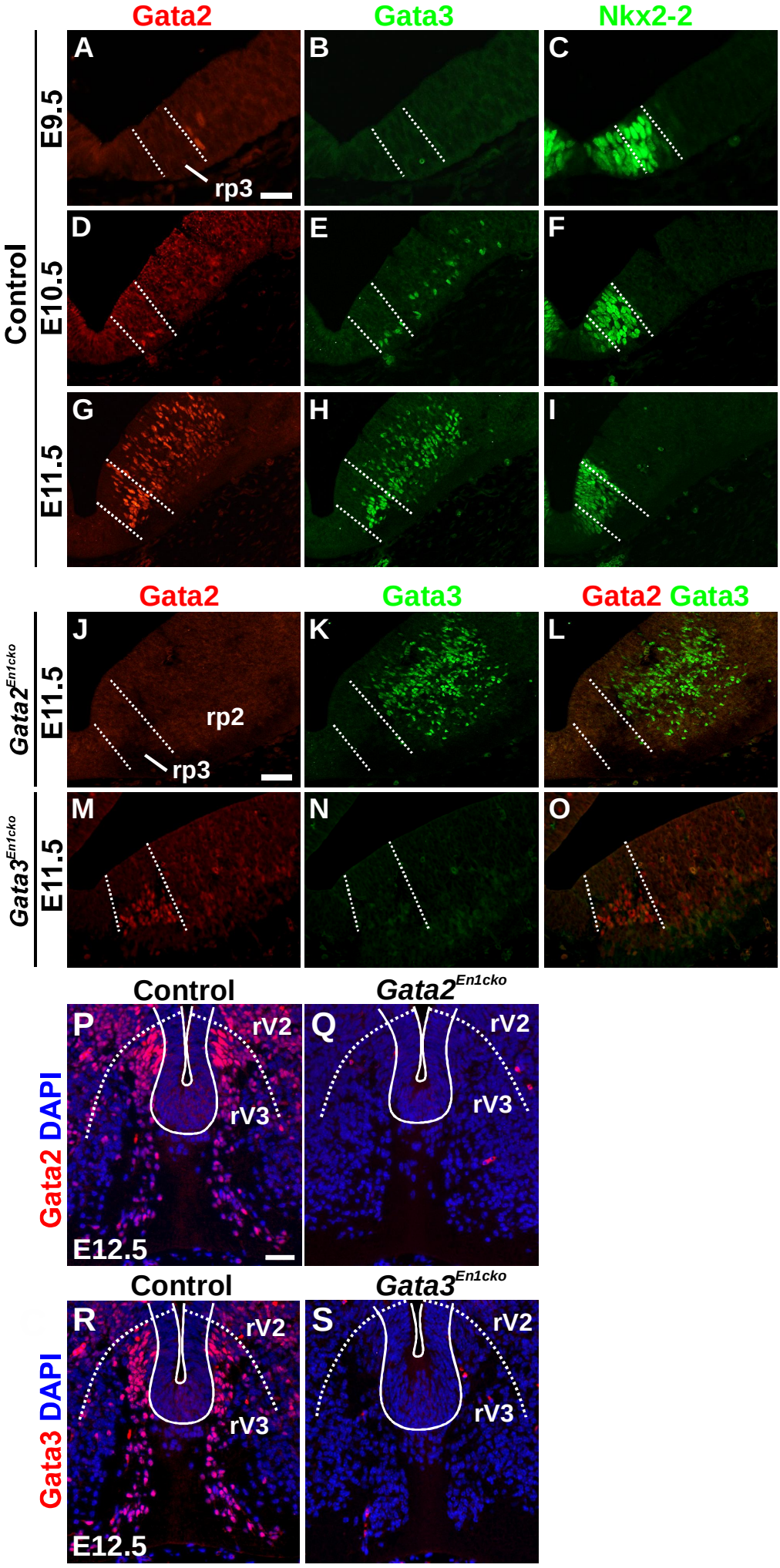

Figure S4. Pattern of expression and conditional inactivation of Gata2 and Gata3 in the ventral r1.

(A-I) IHC analysis of Gata2, Gata3 and Nkx2-2 expression in wild-type embryos at E9.5, E10.5 and E11.5. (J-S) IHC analysis of Gata2 and Gata3 expression in *Gata2*^{En1cko} and *Gata3*^{En1cko} embryos at E11.5 and E12.5. Dotted lines indicate the borders of rp3/rV3 domain. Scale bars: 50 µm (A,J), 100 µm (P).

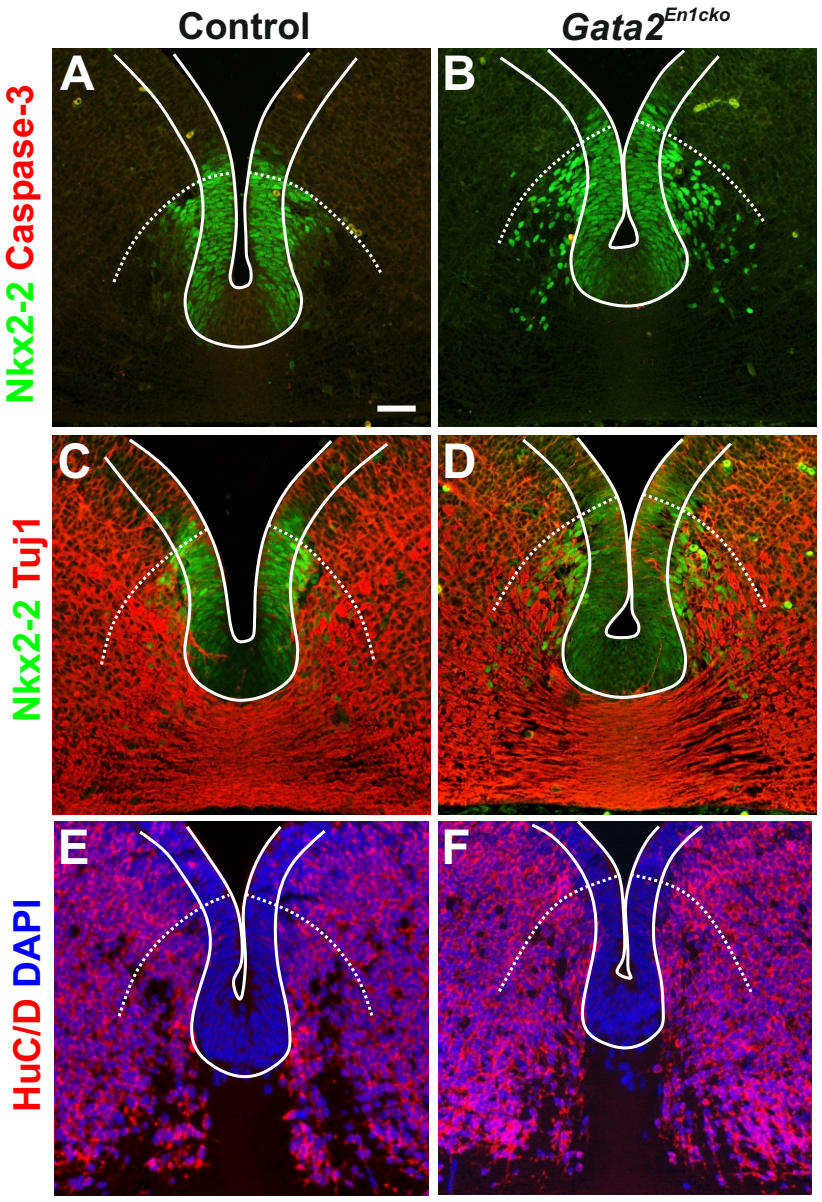

Figure S5. Generation of neural precursors in the ventral r1 in *Gata2*^{En1cko} embryos.

(A,B) IHC analysis of Nkx2-2 and Caspase-3, (C,D) IHC analysis of Nkx2-2 and Tuj1, (E,F) IHC analysis of HuC/D expression in post-mitotic neural precursors show no differences between E12.5 wild-type and *Gata2*^{En1cko} mutants. Dotted lines mark the border between domains rp3/rV3 and rpMN/rVMN. Scale bar: 100 µm.

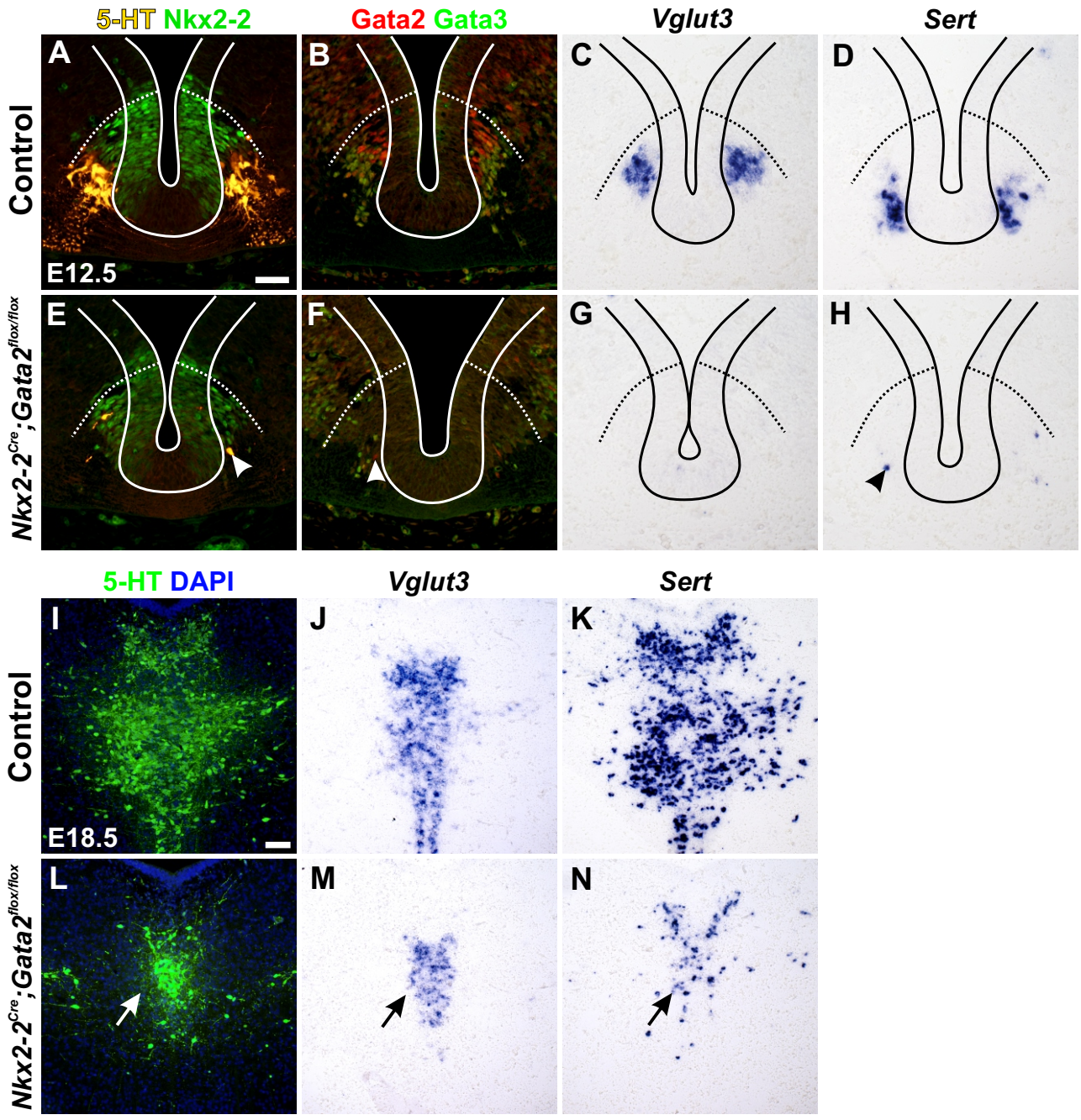

Figure S6. Serotonergic neuron development in *Nkx2-2^{Cre};Gata2$^{flox/flox}$*embryos.

(A,E) IHC of 5-HT and Nkx2-2, (B,F) IHC of Gata2 and Gata3, (C,G) ISH of *Vglut3* and (D,H) ISH analysis of *Sert* expression in E12.5 control and *Nkx2-2^{Cre};Gata2$^{flox/flox}$*embryos. (I-L) IHC of 5-HT, (J,M) ISH of *Vglut3*, and (K,N) ISH analysis of *Sert* expression in E18.5 control and *Nkx2-2^{Cre};Gata2$^{flox/flox}$*embryos. Arrowheads show remaining positive cells, arrows indicate reduced cell populations in the *Nkx2-2^{Cre};Gata2$^{flox/flox}$* embryos . Dotted lines mark the border between rp3/rV3 and rpMN/rVMN. Scale bars: 50 µm (A) 100 µm (I).

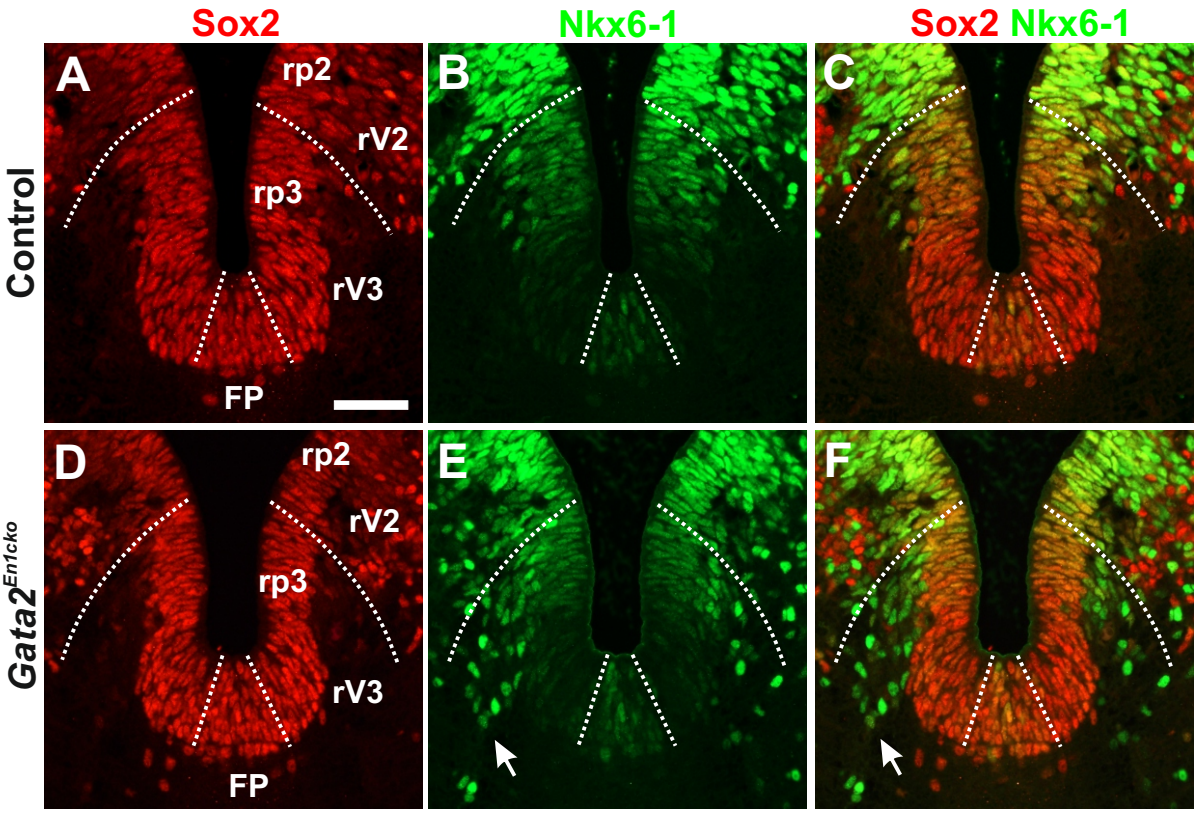

Figure S7. Expression of Nkx6-1 in postmitotic precursors in rV3 domain in *Gata2*^{En1cko}.

(A-F) IHC of Sox2 and Nkx6-1 expression in the ventral r1 of E12.5 control and *Gata2*^{En1cko} embryos. Arrows indicate Nkx6-1 expression in the post-mitotic precursors negative for Sox2 expression. Note also weak expression of Nkx6-1 in the medial floor plate (FP). Dotted lines lineate the dorsal border of rp3 and rV3 domains and Nkx6-1^{+} progenitors in the floor plate. Scale bar: 50 µm.

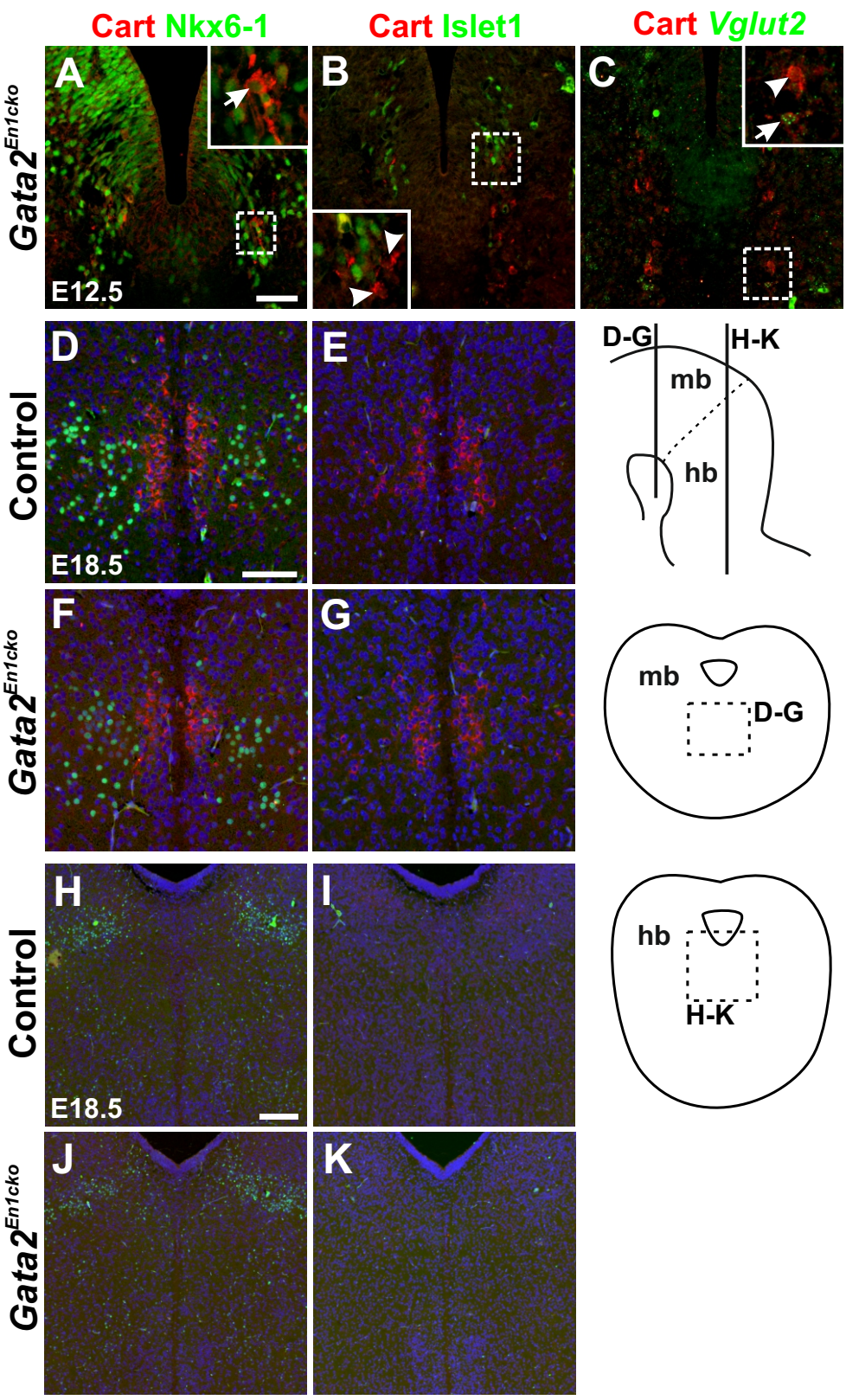

Figure S8. Expression of Cart in *Gata2*^{En1cko} embryos.

(A-C) Cart IHC with Nkx6-1 IHC, Islet1 IHC, and *Vglut2* ISH in E12.5 *Gata2*^{En1cko}. (D-G) Cart, Nkx6-1 and Islet1 IHC in the midbrain preEdinger-Westphal nucleus of E18.5 control and *Gata2*^{En1cko} embryos. (H-K) Cart, Nkx6.1 and Islet1 IHC in the dorsal raphe region of E18.5 control and *Gata2*^{En1cko} embryos. Arrows indicates co-expression, arrowheads mark Cart positive cells that are Islet1 or *Vglut2* negative. mb, midbrain; hb, hindbrain. Scale bars: 50 µm (A), 100 µm (D).

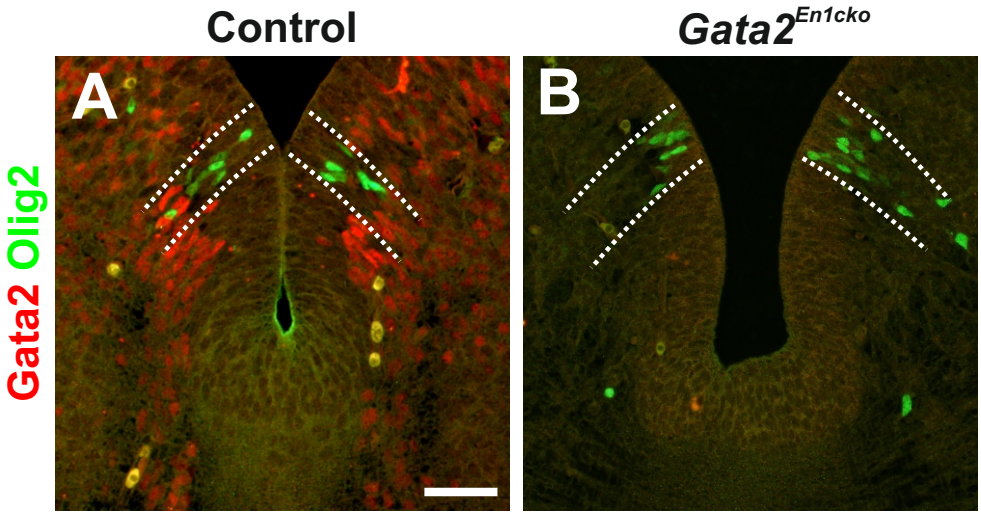

Figure S9. Expression of Olig2 in the *Gata2*^{En1cko} embryos.

(A-B) IHC of Gata2 and Olig2 in the ventral r1 of E12.5 control and *Gata2*^{En1cko} embryos. Dotted lines indicate the rpMN domain. Scale bar: 50 µm.

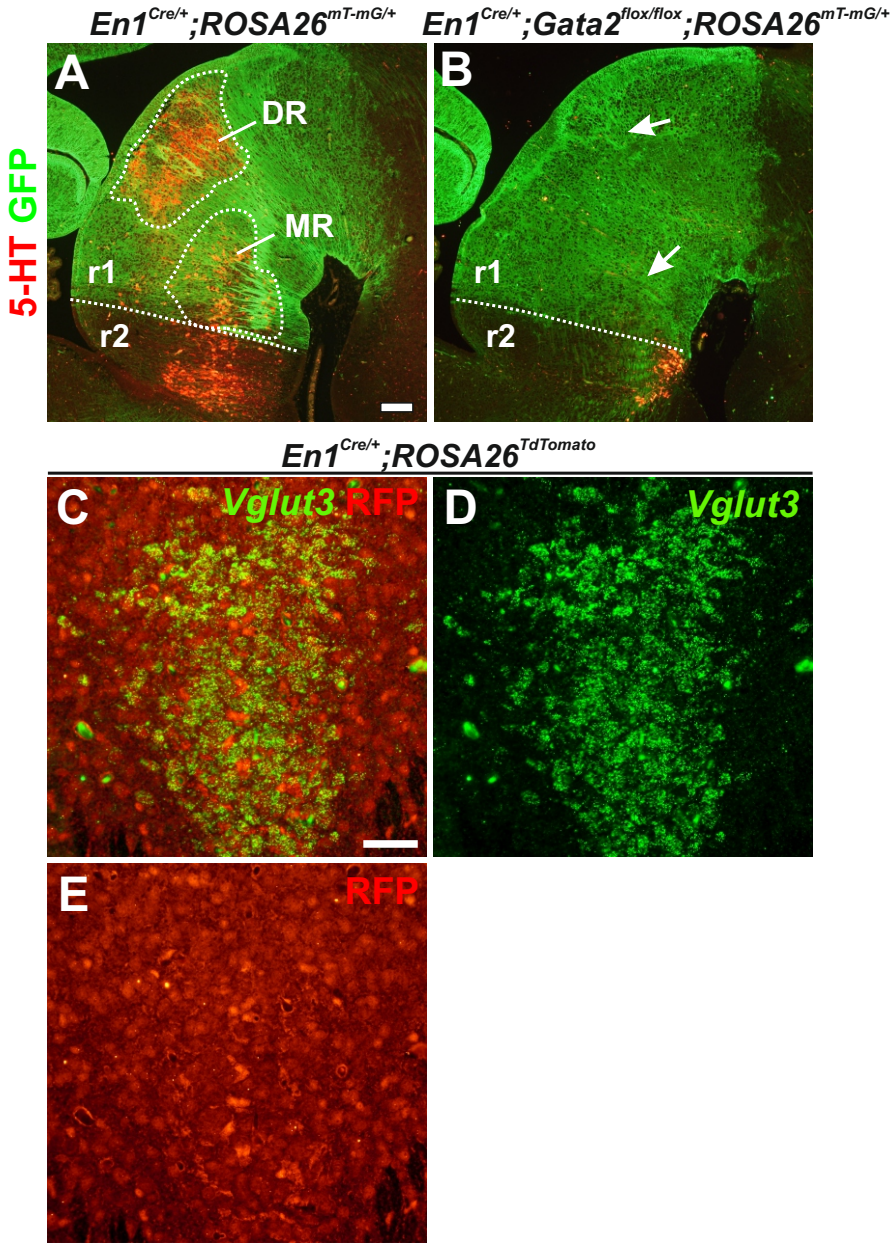

Figure S10. Loss of the r1-derived serotonergic neurons in the *Gata2*^{En1cko} brain.

(A,B) 5-HT and GFP IHC on sagittal sections of E18.5 control and *Gata2*^{En1cko} embryos carrying a *ROSA26*$^{mT\text{-}mG}$ allele (exact genotypes indicated at the top). *En1*^{Cre} activated GFP expression marks r1 and midbrain derived cells. Arrows indicate loss of 5-HT positive cells in the dorsal raphe and median raphe. Straight dotted lines indicate border between the r1 and r2. (C-E) *Vglut3* ISH and RFP IHC in *En1*^{Cre};*ROSA26*^{TdTomato} embryos, where RFP marks the r1 and midbrain derived cells. r1, rhombomere 1; r2, rhombomere 2; DR, dorsal raphe; MR, median raphe. Scale bars: 50 µm (C') 200 µm (A).

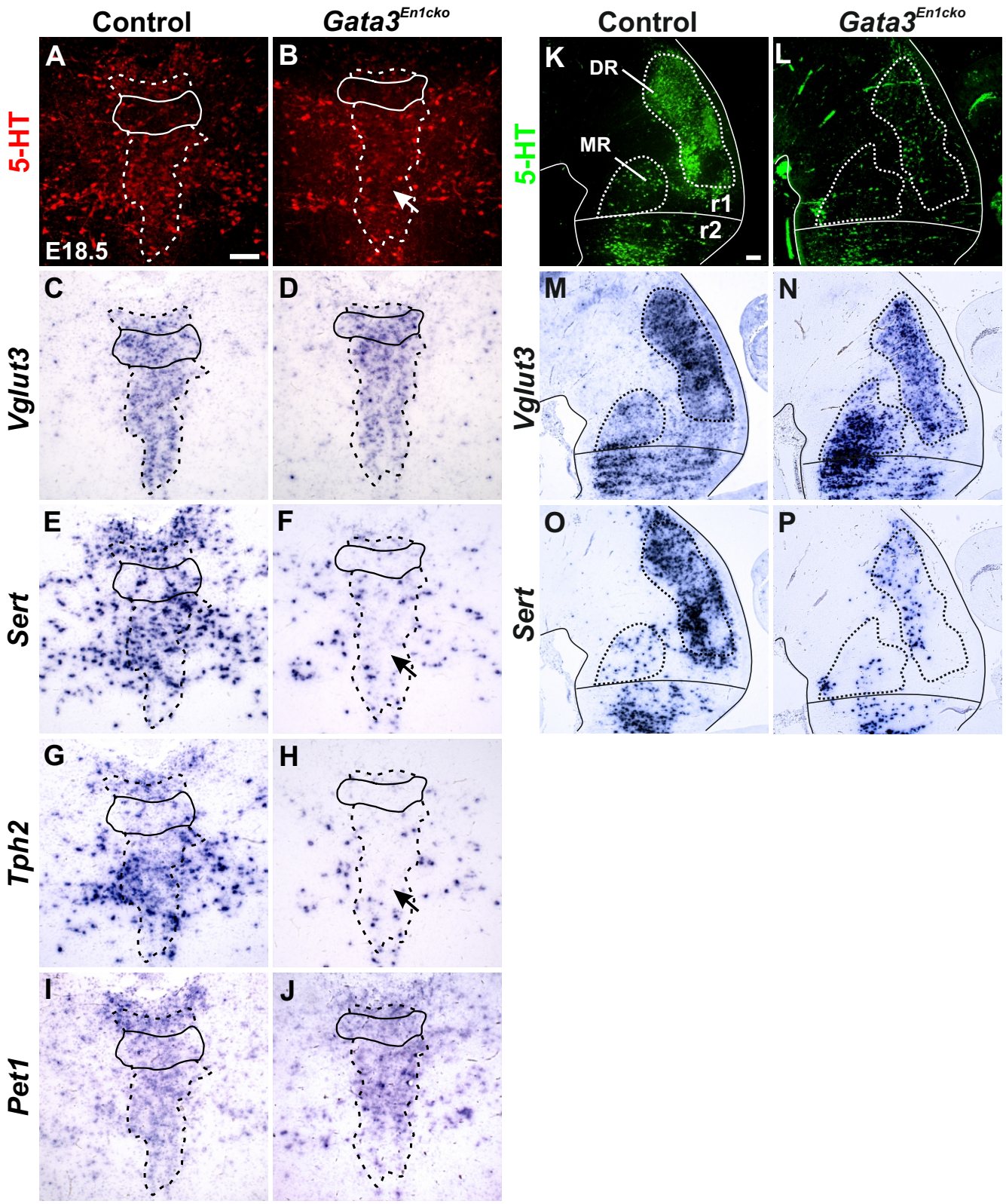

Figure S11. Loss of serotonergic markers in the medial region of the dorsal raphe in the *Gata3*^{En1cko} brain.

Coronal (A-J) and sagittal (K-P) sections of control and *Gata3*^{En1cko} embryos at E18.5. (A-P) IHC analysis of 5-HT and ISH analysis of *Vglut3*, *Sert*, *Tph2* and *Pet1* expression on parallel sections. Non-regular dotted lines indicate the expression area of *Vglut3*, while continuous lines mark the *Vglut3* positive but mostly 5-HT negative area in (A-J) and a border between r1-r2. Arrows indicate loss of expression. r1, rhombomere 1; r2, rhombomere 2; DR, dorsal raphe; MR, median raphe. Scale bar: 100 µm (A), 200 µm (K).

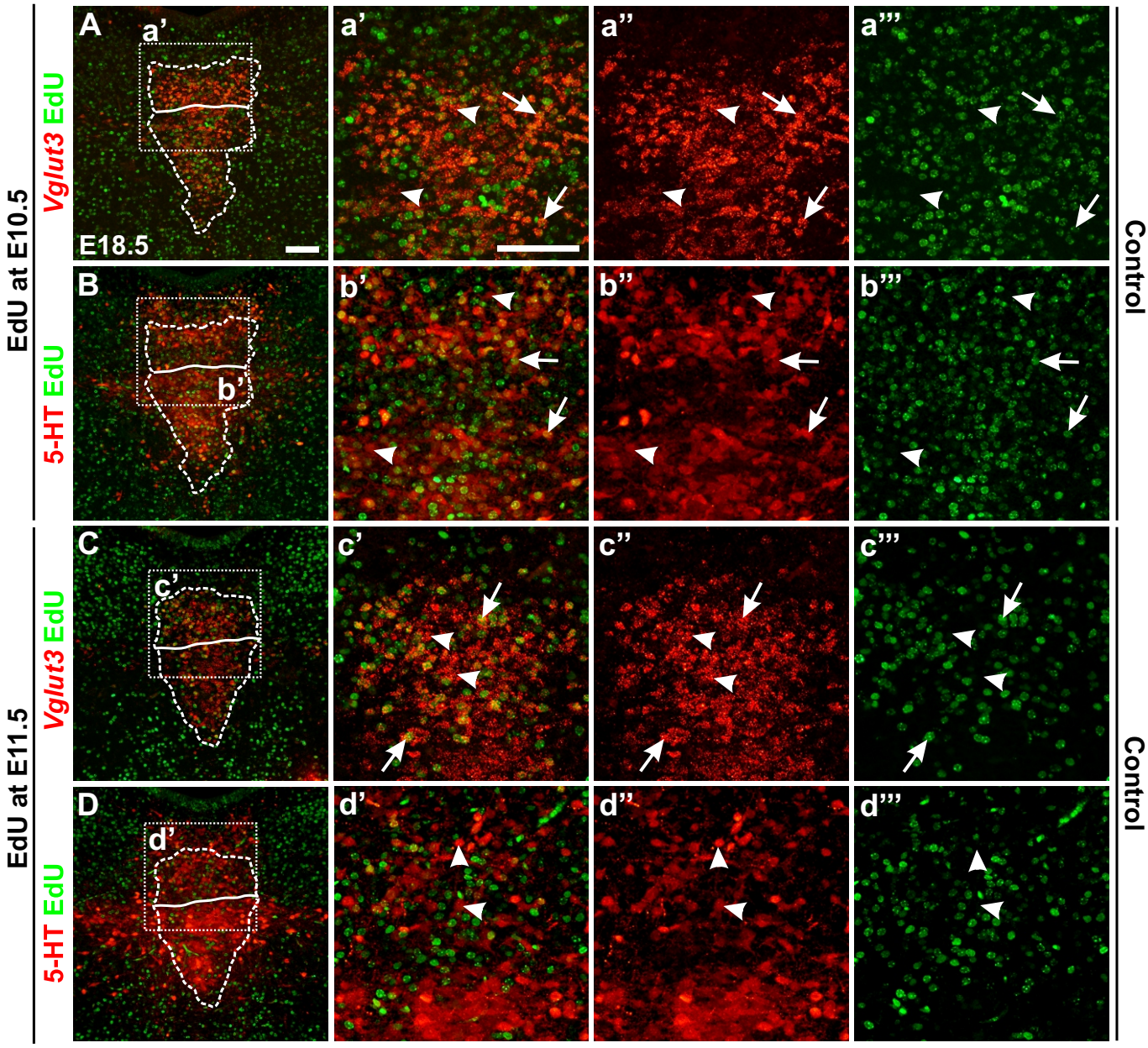

Figure S12. EdU labelling of dorsal raphe progenitors at E10.5 and E11.5.

(A-D) *Vglut3* ISH and 5-HT IHC combined with EdU detection on coronal sections of E18.5 wild-type dorsal raphe with higher magnification pictures in (a'-d'''). The embryos were labelled with EdU at E10.5 or at E11.5. Arrows indicate double positive cells for EdU and *Vglut3* or 5-HT, arrowheads mark cells that are EdU positive but negative for *Vglut3* or 5-HT. Dotted lines indicate the expression area of *Vglut3*, while continuous line marks the border of the region containing cells expressing *Vglut3* but not 5-HT. Scale bars: 100 μm.

Table S1. List of primary and secondary antibodies.

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Table S2. List of *in situ* probes.

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Table S3. Gene ontology analysis of the genes down-regulated in the *Gata2*^{En1cko} mutants. Illumina GeneChip cDNA microarray –based profiling of gene expression in the ventral r1 of E12.5 wild-type and *Gata2*^{En1cko} mutants revealed 20 genes expressed at a lower level in *Gata2*^{En1cko} (adjusted P-value <0.05). (A-C) GOrilla analyses of the gene set down-regulated in the *Gata2*^{En1cko} mutants. Statistically significantly enriched gene ontology terms include several terms specific for neuronal functions and serotonergic neurotransmission. (D) Expression of the genes altered in *Gata2*^{En1cko} in different serotonergic neuron subgroups (data from Okaty et al., 2015). High fold-change values in *Gata2*^{En1cko} seem to be frequent in genes that are highly expressed in R1DR/MR.

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