

RESEARCH ARTICLE

Otx2 cell-autonomously determines dorsal mesencephalon versus cerebellum fate independently of isthmus organizing activity

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ABSTRACT

During embryonic development, the rostral neuroectoderm is regionalized into broad areas that are subsequently subdivided into progenitor compartments with specialized identity and fate. These events are controlled by signals emitted by organizing centers and interpreted by target progenitors, which activate superimposing waves of intrinsic factors restricting their identity and fate. The transcription factor Otx2 plays a crucial role in mesencephalic development by positioning the midbrain-hindbrain boundary (MHB) and its organizing activity. Here, we investigated whether Otx2 is cell-autonomously required to control identity and fate of dorsal mesencephalic progenitors. With this aim, we have inactivated Otx2 in the Pax7⁺ dorsal mesencephalic domain, previously named m1, without affecting MHB integrity. We found that the Pax7⁺ m1 domain can be further subdivided into a dorsal Zic1⁺ m1a and a ventral Zic1[−] m1b sub-domain. Loss of Otx2 in the m1a (Pax7⁺ Zic1⁺) sub-domain impairs the identity and fate of progenitors, which undergo a full switch into a coordinated cerebellum differentiation program. By contrast, in the m1b sub-domain (Pax7⁺ Zic1[−]) Otx2 is prevalently required for post-mitotic transition of mesencephalic GABAergic precursors. Moreover, genetic cell fate, BrdU cell labeling and Otx2 conditional inactivation experiments indicate that in Otx2 mutants all ectopic cerebellar cell types, including external granule cell layer (EGL) precursors, originate from the m1a progenitor sub-domain and that reprogramming of mesencephalic precursors into EGL or cerebellar GABAergic progenitors depends on temporal sensitivity to Otx2 ablation. Together, these findings indicate that Otx2 intrinsically controls different aspects of dorsal mesencephalic neurogenesis. In this context, Otx2 is cell-autonomously required in the m1a sub-domain to suppress cerebellar fate and promote mesencephalic differentiation independently of the MHB organizing activity.

KEY WORDS: Otx2, Dorsal mesencephalon, Cerebellum, Progenitor fate, Mouse

INTRODUCTION

A wealth of data have indicated that the specification of regional identities and sub-regional domains along the anterior-posterior (A-P) and dorsal-ventral (D-V) axes of the neural tube is induced by signaling molecules produced by organizing centers and interpreted by target progenitor domains. The nature and efficiency of the signaling inducer(s) and the competence of the responding tissue(s) provide precursor cells with the activation of a specific differentiation program on which neuronal identity, fate and functioning largely depend (Rubenstein et al., 1998; Lumsden and Krumlauf, 1996; Jessell, 2000; Briscoe and Ericson, 2001). The activation of specific transcription factors at precise developmental stages generates subsequent waves of information gradually restricting identity and fate of progenitor cells. This molecular strategy thus provides specific sub-programs of cellular differentiation to proliferating and post-mitotic progenitors. In this context, transplantation as well as molecular and genetic studies indicated that development of the midbrain and cerebellum can be regulated by a ring of organizer cells located at the midbrain-hindbrain border (MHB) and called the isthmus organizer (ISO) (Alvarado-Mallart, 1993; Nakamura, 2001; Sato et al., 2001; Martinez et al., 1991; Hatten et al., 1997; Wurst and Bally-Cuif, 2001; Sillitoe and Joyner, 2007; Liu and Joyner, 2001a). The signaling molecule Fgf8 is expressed by the ISO, can induce ectopic midbrain and cerebellum and is required for their development (Crossley et al., 1996; Martinez et al., 1999). Importantly, among the transcription factors expressed in the midbrain-hindbrain region, Otx2 is crucial to define the positioning of the ISO at the MHB and specify the mesencephalic territory (Acampora et al., 1997; Broccoli et al., 1999; Millet et al., 1999; Simeone, 2000; Wurst and Bally-Cuif, 2001; Liu and Joyner, 2001b; Rhinn and Brand, 2001; Simeone et al., 2002; Puelles et al., 2003; Martinez-Barbera et al., 2001; Nakamura, 2001; Foucher et al., 2006; Sillitoe and Joyner, 2007). In addition, Otx2 is also required for the neurogenesis of ventral progenitor domains fated to generate dopaminergic and red nucleus neurons (Puelles et al., 2003; Prakash et al., 2006; Prakash et al., 2009; Omodei et al., 2008; Di Salvio et al., 2010; Di Giovannantonio et al., 2013). In this context, it has been reported that Otx2 may directly interact with the Groucho co-repressor Tle4 (also known as Grg4) and the TALE homeobox gene Meis2 (Puelles et al., 2004; Heimbucher et al., 2007; Agoston and Schulte, 2009; Agoston et al., 2012), and it has been proposed that in chick embryos Meis2, which is required for neurogenesis of the dorsal mesencephalon, performs this action through interaction with Otx2 (Agoston and Schulte, 2009). Further studies identified seven progenitor domains along the mesencephalic D-V axis (Nakatani et al., 2007; Kala et al., 2009) and showed that in the mesencephalon, including the dorsal mesencephalic 1 (m1) domain, Helt and Ngn1

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(Neurogl – Mouse Genome Informatics) are required for the differentiation of GABAergic and glutamatergic neurons, respectively (Nakatani et al., 2007; Guimera et al., 2006), and that *Gata2* is a GABAergic determinant expressed in early post-mitotic precursors and required for their differentiation (Kala et al., 2009). We have now concentrated our efforts on the role of *Otx2* in the m1 domain and investigated whether *Otx2* is cell-autonomously required for regional specification and/or differentiation of these progenitors. To this aim, we have employed a mouse model carrying a *Dbx1*-driven *Ires Cre* recombinase (*Dbx1^{ICre/+}*), which inactivates *Otx2* in progenitors of the m1 domain without affecting MHB integrity (Bielle et al., 2005; Causeret et al., 2011). Based on the distinctive expression of *Zic1*, we observed that the m1 (*Pax7⁺*) domain may be divided into m1a and m1b sub-domains. We found that lack of *Otx2* remarkably affected the identity and fate of the m1a (*Zic1⁺ Pax7⁺*) sub-domain, which underwent a dramatic change of its differentiation program resulting in a mesencephalic switch into a coordinated program of cerebellar neurogenesis. The ventral m1b (*Zic1⁻ Pax7⁺*) sub-domain exhibited a severe impairment in the mesencephalic GABAergic differentiation. Together, these data indicate that *Otx2* is a novel intrinsic determinant differentially controlling the neurogenesis of dorsal mesencephalic progenitor sub-domains. Moreover, *Otx2*, cell-autonomously and independently of the MHB organizing activity, determines dorsal mesencephalon versus cerebellum fate.

RESULTS

Otx2 expression in the dorsal mesencephalon

Previous studies have provided relevant data on the molecular determinants controlling the neurogenesis of specific progenitor domains distributed along the mesencephalic D-V axis (Nakatani et al., 2007; Kala et al., 2009). Here, we concentrated our analysis on the role of *Otx2* in the dorsal mesencephalic m1 domain, which generates glutamatergic and GABAergic neurons of the tectum (Nakatani et al., 2007; Kala et al., 2009). During embryonic development, *Otx2* was expressed in virtually all progenitors of the m1 domain. In particular, at embryonic day (E) 11 and E13.5 *Otx2* was co-expressed in m1 progenitors with *Zic1*, *Pax7*, *Dbx1*, *Mash1* (Ascl1 – Mouse Genome Informatics), *Helt* and *Ngn1* (supplementary material Fig. S1A-F') in GABAergic early post-mitotic progenitors with *Gata2* (supplementary material Fig. S1G,G'), and in GABAergic post-mitotic neurons with *Lhx1*, *Pax7* and *Gad65* (*Gad2* – Mouse Genome Informatics) (supplementary material Fig. S1B,B',H,H',J,J'; data not shown). By contrast, *Otx2* expression was excluded from the large majority of post-mitotic glutamatergic precursors expressing *Pou4f1* (also known as *Brn3a*) and *vGlut2* (*Slc17a6* – Mouse Genome Informatics) (supplementary material Fig. S1I,I',K,K'). This neuronal restriction was maintained by *Otx2* until the adult stage (supplementary material Fig. S1L-O'; data not shown). Interestingly, this marker analysis suggests that, at least on the basis of *Zic1* expression, the m1 domain may be subdivided in the m1a (*Zic1⁺*) and m1b (*Zic1⁻*) sub-domains.

Otx2 inactivation by *Dbx1*-driven *ICre* recombinase does not affect the integrity of the MHB

Little is known about the cell-autonomous role of *Otx2* as a determinant of dorsal mesencephalic neurogenesis nor its role as a territorial factor directing mesencephalic competence in responding to inducing properties provided by the ISO. The main problem in addressing these questions is the fact that early inactivation of *Otx2* along the A-P axis of the mesencephalon

invariably generates an anterior shift of the ISO. As *Dbx1* was expressed in the m1 progenitor domain (supplementary material Fig. S1C,C') and excluded from the region close to the MHB (Fig. 1C,C',I,I', arrows), we employed in this study the *Dbx1^{ICre/+}* deleter strain (Bielle et al., 2005; Causeret et al., 2011) to conditionally inactivate *Otx2*. Expression analysis in E10.5 *Dbx1^{ICre/+}* and *Dbx1^{ICre/+};Otx2^{lox/lox}* embryos showed that, according to the expression of *Dbx1* and *Cre* genes (Fig. 1B-C',E-F',H-I'), *Otx2* protein was ablated in the m1 domain, but retained in proximity of the MHB, in the mesencephalic territory ventral to the m1 domain and in the roof plate (Fig. 1A',D',G'). To assess whether the residual *Otx2* protein was able to prevent MHB abnormalities, we analyzed the expression of typical MHB markers, such as *Fgf8*, *Gbx2*, *Pax2* and *Wnt1*. Compared with *Dbx1^{ICre/+}* control embryos, in *Dbx1^{ICre/+};Otx2^{lox/lox}* mutants no abnormalities were detected at the MHB region for *Fgf8*, *Gbx2*, *Pax2* and *Wnt1* (Fig. 1L-W). The same result was confirmed also at E12.5 (data not shown). Therefore, these data indicate that *Dbx1^{ICre/+};Otx2^{lox/lox}* mice may represent a relevant model in which to study for the first time the cell-autonomous role of *Otx2* in the dorsal mesencephalon without the interference of molecular and anatomical abnormalities affecting the integrity of the MHB.

Otx2 is required for differentiation and fate of m1 progenitors

Next, we performed a detailed marker analysis to assess whether identity and initial fate of m1 progenitors was affected in embryos lacking *Otx2*. First, we confirmed in *Dbx1^{ICre/+};Otx2^{lox/lox}* mutants the full inactivation of *Otx2* in the territory normally expressing *Dbx1*-driven *Cre* recombinase (Fig. 2A-C'). We found that, in contrast to E10.5 mutants (Fig. 1), at E11.5 the expression of *Dbx1* disappeared from the m1a sub-domain of *Dbx1^{ICre/+};Otx2^{lox/lox}* embryos whereas it was retained in the m1b region (Fig. 2D', white arrow), which corresponded to the *Pax7⁺ Zic1⁻* region (Fig. 2G'). Moreover, in E11.5, E12.5 and E13.5 *Otx2* mutant embryos, *Zic1* expression was expanded ventrally whereas *Pax7* was gradually downregulated in most of the *Zic1⁺* domain (Fig. 2G'-I'). Thus, these data suggest that *Otx2* is required both to maintain *Pax7* and *Dbx1* expression in the m1a sub-domain and to prevent its ventral expansion at the expense of the m1b sub-domain. Previous studies showed that the m1 domain generated GABAergic and glutamatergic neurons controlled respectively by specific determinants: GABAergic fate was initiated by progenitors expressing *Mash1* and *Helt*, which were required for post-mitotic activation of *Gata2* and *Lhx1*; glutamatergic fate was instead initiated by *Ngn1⁺* progenitors which post-mitotically activated *Pou4f1* (Nakatani et al., 2007; Kala et al., 2009; Guimera et al., 2006). Compared with E11.5, E12.5 and E13.5 control embryos, those lacking *Otx2* retained the expression of *Mash1* and failed to activate *Helt* in the m1a sub-domain and *Gata2* in both the m1 sub-domains (Fig. 2J-O'); by contrast, *Lhx1⁺* neurons were prevalently concentrated in the m1a sub-domain of *Otx2* mutants (Fig. 2P-R'), co-expressed *Gad65* (Fig. 2S', arrow) but lacked *Pax7* (Fig. 2G-I'; data not shown). In the same mutants, *Ngn1* expression was apparently normal in the m1b sub-domain (Fig. 2D'-F', white arrows) whereas in the m1a sub-domain the number of *Ngn1⁺* progenitors was reduced and confined to the dorsal half of this sub-domain (Fig. 2D'-F', yellow arrows). At E11, glutamatergic *Pou4f1⁺* neurons were moderately reduced in number all along the m1 domain, but, subsequently, their number was remarkably affected only in the m1a sub-domain (Fig. 2M-R'). Accordingly, at E13.5 *Pou4f1⁺ vGlut2⁺* neurons were restricted to the m1b sub-

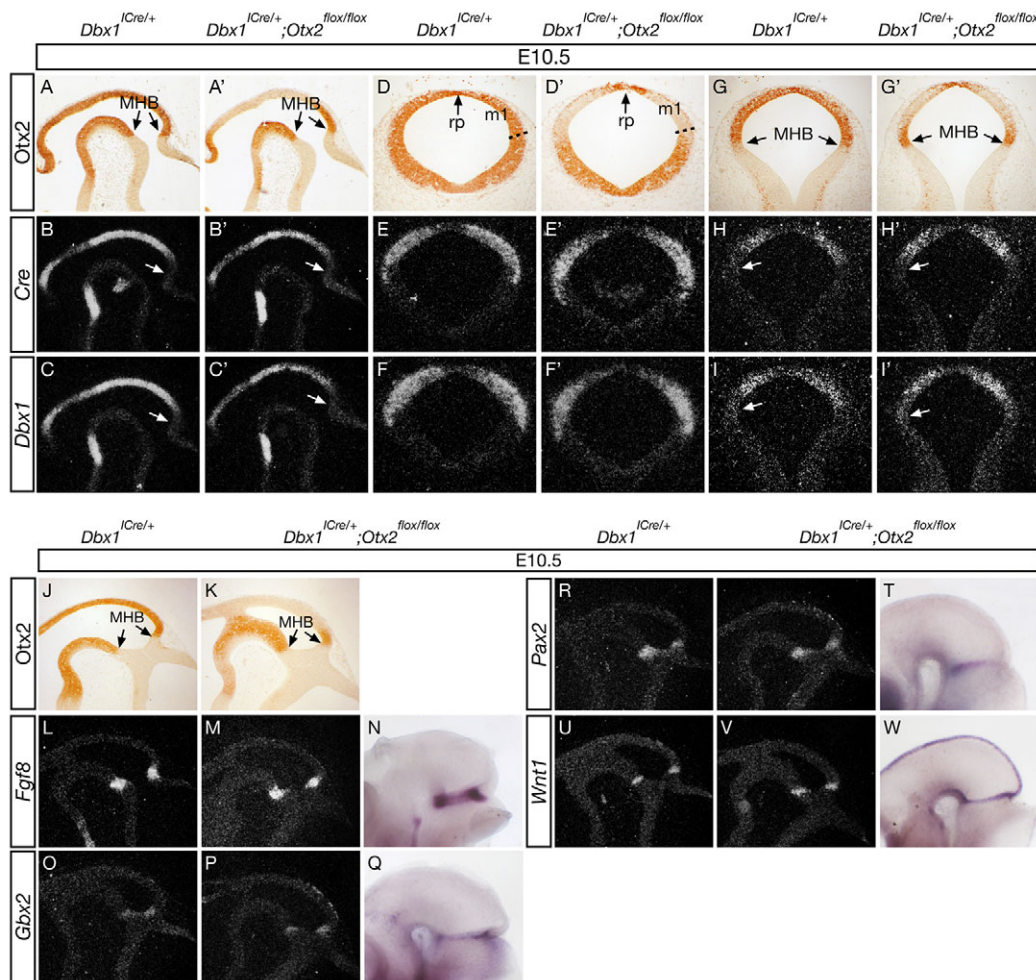


Fig. 1. Otx2 inactivation by *Dbx1*-driven Ires Cre activity and MHB integrity in *Dbx1*^{Icre/+}; *Otx2*^{flox/flox} embryos. (A-I') Immunohistochemistry and RNA *in situ* hybridization experiments performed on E10.5 *Dbx1*^{Icre/+} and *Dbx1*^{Icre/+}; *Otx2*^{flox/flox} sagittal (A-C') and frontal (D-I') sections with the Otx2 antibody (A,A',D,D',G,G'), the Cre probe (B,B',E,E',H,H') or the *Dbx1* probe (C,C',F,F',I,I') show that Otx2 is not ablated in proximity of the MHB and roof plate where indeed Cre and *Dbx1* are not expressed (B-C',H-I', arrows). (J-W) Immunohistochemistry and RNA *in situ* hybridization experiments performed on E10.5 *Dbx1*^{Icre/+} and *Dbx1*^{Icre/+}; *Otx2*^{flox/flox} embryos with the Otx2 antibody (J,K), or the *Fgf8* (L-N), *Gbx2* (O-Q), *Pax2* (R-T) or *Wnt1* (U-W) probes show that in *Dbx1*^{Icre/+}; *Otx2*^{flox/flox} embryos Otx2 is not ablated in proximity of the MHB region, which, indeed, retains an apparently normal molecular code. The dashed line in D,D' points to the presumptive ventral border of the m1 domain. MHB, midbrain-hindbrain boundary; rp, roof plate; m1, mesencephalic domain 1.

domain (Fig. 2T', arrow). Thus, this marker analysis indicates that in the absence of Otx2 the m1 differentiation program controlling GABAergic and glutamatergic fates is severely affected. Indeed, apparently in contrast with previous studies (Nakatani et al., 2007; Kala et al., 2009; Guimera et al., 2006), in Otx2 mutants, loss of Helt and Gata2 in the m1a sub-domain did not prevent GABAergic differentiation; rather, the generation of GABAergic neurons was increased in this sub-domain and severely diminished in the m1b sub-domain. Conversely, Pou4f1⁺ vGlut2⁺ neurons were concentrated in the post-mitotic area of the m1b sub-domain. These data suggest that Otx2 cell-autonomously controls the neurogenesis of the m1 domain. Moreover, these data led us to hypothesize that in Otx2 mutants the differentiation program of the m1a sub-domain might have been converted into that of a different brain region not requiring Otx2, Helt or Gata2 to promote GABAergic differentiation.

Otx2 prevents dorsal mesencephalon into cerebellum fate switch

We reasoned that abnormalities described in the m1a sub-domain might be due to the activation of a differentiation program normally suppressed by Otx2 in the mesencephalon. On this basis, we evaluated the expression of previous markers in the developing cerebellum, which is originated by a territory initially not expressing Otx2. Otx2 was indeed expressed until E12.5 in the choroid plexus (chp), and from E13.5 onwards also in the rhombic lip (supplementary material Fig. S2A,B). In adult mice, Otx2 was

expressed in the internal granule cell layer (IGL) of posterior cerebellar lobules (Frantz et al., 1994) (data not shown). Pax6 expression was detected at E12.5 and E13.5 in the emerging external granule cell layer (EGL), rhombic lip and ventricular/sub-ventricular zone (VZ/SVZ) close to it (supplementary material Fig. S2C,D). As for the other markers, Ngn1, Mash1 and Zic1 were detected at E12.5 and E13.5 in the VZ/SVZ (supplementary material Fig. S2E-H); Zic1 was also expressed in the emerging EGL and rhombic lip (supplementary material Fig. S2E,F); Pax7 was expressed in the VZ/SVZ and downregulated after E13.5 (supplementary material Fig. S2C,D,O-R; see also Fig. 3O); and Lhx1 was expressed in post-mitotic precursors of Purkinje cells (Zhao et al., 2007; Morales and Hatten, 2006) and in most of those expressing Pax2 and fated to generate GABAergic interneurons (Maricich and Herrup, 1999; Weisheit et al., 2006; Glassmann et al., 2009) (supplementary material Fig. S2E,F,I,J). Importantly, Helt, Gata2, *Dbx1* and Pou4f1 were not detected in the developing cerebellum (supplementary material Fig. S2K-R). It is noteworthy that in the cerebellum Ngn1 was expressed in precursors of Purkinje cells and GABAergic interneurons whereas in the m1 domain it was restricted to glutamatergic progenitors. Therefore, our data indicate that the m1a sub-domain of Otx2 mutants and the normal cerebellum exhibit a similar expression profile for Zic1, Otx2, *Dbx1*, Pax7, Helt, Gata2, Lhx1 and Pou4f1. Thus, we investigated whether in the absence of Otx2 a mesencephalic into a cerebellar fate switch may occur in the m1a sub-domain. We found that, like in the cerebellum, in the dorsal mesencephalon of

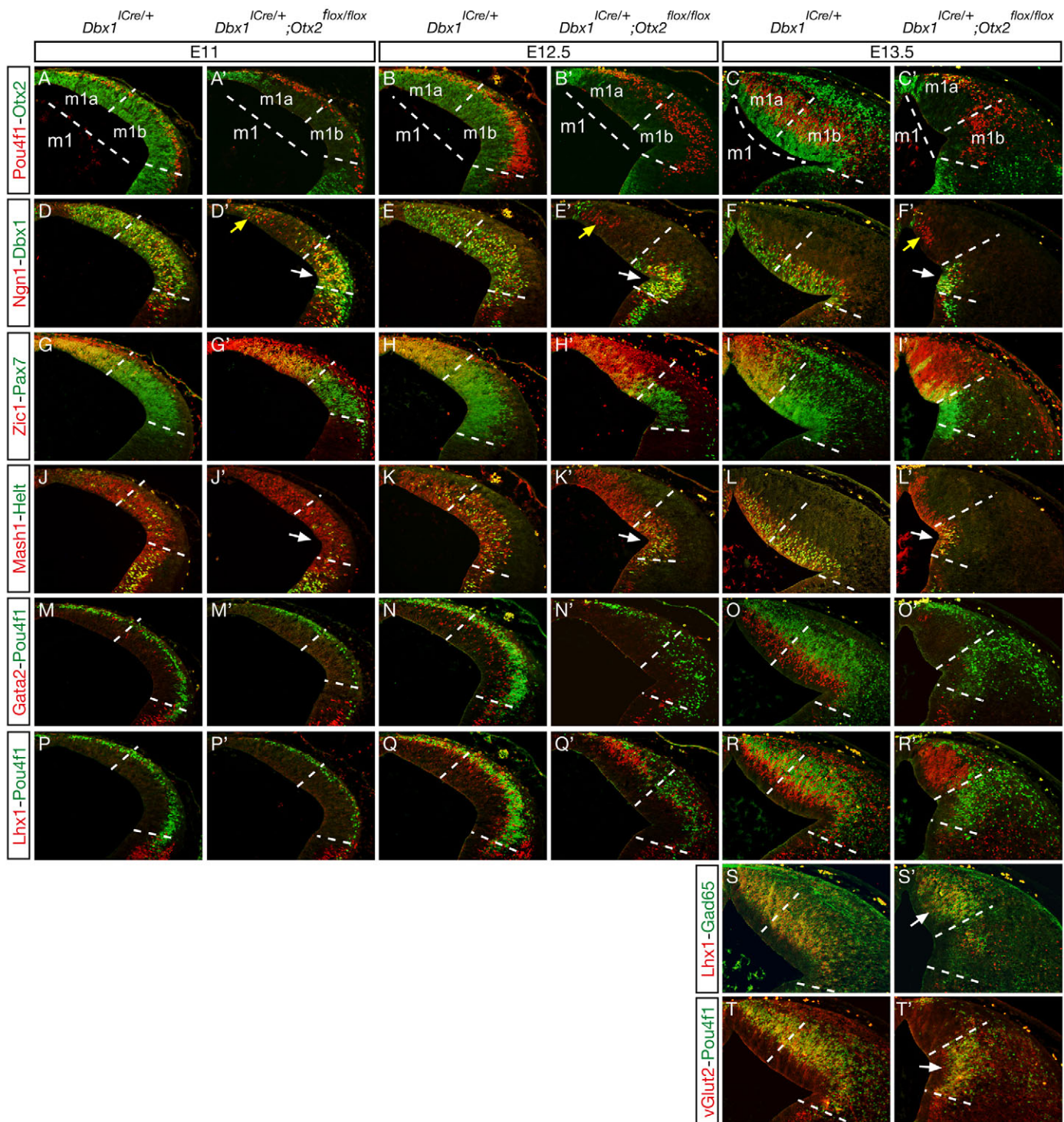


Fig. 2. Loss of Otx2 remarkably affects identity and differentiation of m1 progenitors. Immunohistochemistry experiments performed at E11, E12.5 and E13.5 on *Dbx1*^{ICre/+} and *Dbx1*^{ICre/+};*Otx2*^{flox/flox} embryos with Pou4f1 and Otx2 (A-C'), Ngn1 and Dbx1 (D-F'), Zic1 and Pax7 (G-I'), Mash1 and Helt (J-L'), Gata2 and Pou4f1 (M-O'), Lhx1 and Pou4f1 (P-R'), Lhx1 and Gad65 (S,S') or vGlut2 and Pou4f1 (T,T'). The white arrows in D'-F' and J'-L' point respectively to the expression of Dbx1 and Ngn1, and Helt and Mash1 in the m1b sub-domain; the yellow arrows in D'-F' point to the Ngn1 residual expression in the m1a sub-domain; and the white arrows in S' and T' respectively to Lhx1⁺ Gad65⁺ GABAergic and Pou4f1⁺ vGlut2⁺ glutamatergic neurons generated in the m1a and m1b sub-domains. Dashed lines delineate regions as marked in A-C'. m1, mesencephalic domain 1; m1a, mesencephalic sub-domain 1a; m1b, mesencephalic sub-domain 1b.

Dbx1^{ICre/+};*Otx2*^{flox/flox} mutant embryos Pax2⁺ cells co-expressing Lhx1 were first detected at E12.5 and more abundantly at E13.5 (Fig. 3A-D), whereas Pax6 was not expressed until E13.5 (Fig. 3E,F). At E15.5, compared with control embryos

(Fig. 3G,J,M), Otx2 mutant embryos showed that Pax6 was activated in the m1a VZ/SVZ and in a layer of cells located in the outermost mesencephalon (Fig. 3H, arrows). This external cell layer was Otx2⁻ and co-expressed the EGL markers Math1 and

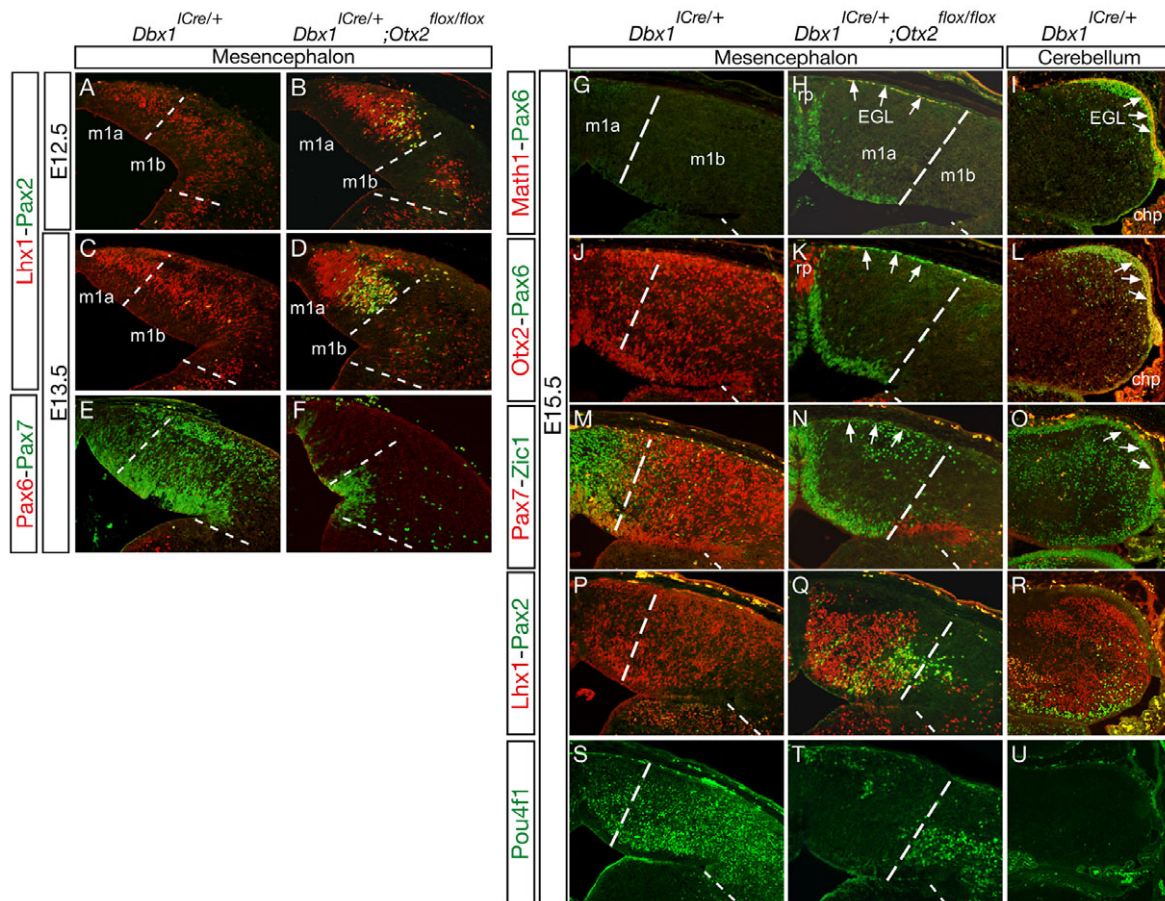


Fig. 3. Loss of Otx2 de-represses a cerebellar-like fate in the m1a sub-domain and affects GABAergic differentiation in the m1b sub-domain.

(A-F) Immunohistochemistry assays performed on *Dbx1*^{Cre/+} and *Dbx1*^{Cre/+}; *Otx2*^{flox/flox} embryos with Lhx1 and Pax2 at E12.5 and E13.5 (A-D) or with Pax6 and Pax7 only at E13.5 (E,F) show that in *Dbx1*^{Cre/+}; *Otx2*^{flox/flox} mutants Lhx1 is expressed prevalently in the m1a sub-domain where a fraction of Lhx1⁺ cells co-expresses Pax2 (B,D), and that Pax6 is not expressed at E13.5 (F). (G-U) Immunohistochemistry assays performed at E15.5 on the mesencephalon and cerebellum of *Dbx1*^{Cre/+} and on the mesencephalon of *Dbx1*^{Cre/+}; *Otx2*^{flox/flox} embryos with Math1 and Pax6 (G-I), Otx2 and Pax6 (J-L), Pax7 and Zic1 (M-O), Lhx1 and Pax2 (P-R) or Pou4f1 (S-U) show that in Otx2 mutants Pax6 is expressed in the m1a VZ/SVZ and the forming EGL; the latter co-expresses Math1 and Zic1 but not Otx2 (H,K,N, white arrows); and Otx2 is retained in the roof plate and a few migrating cells (K). Note that Lhx1 and Pax2 are prevalently confined to the m1a sub-domain (Q), whereas Pou4f1 is confined to the m1b sub-domain (T). Dashed lines delineate regions as marked in upper panels. chp, choroid plexus; EGL, external granule cell layer; m1a, mesencephalic sub-domain 1a; m1b, mesencephalic sub-domain 1b; rp, roof plate.

Zic1 (Fig. 3H,K,N, arrows). In E15.5 Otx2 mutants, Lhx1⁺ and Pax2⁺ neurons were mainly confined to the m1a post-mitotic sub-domain (Fig. 3Q), whereas Pax7 was restricted to the VZ/SVZ of the m1b sub-domain (Fig. 3N) where the generation of glutamatergic Pou4f1⁺ neurons was predominant (Fig. 3T). Together, these expression profiles reveal close similarity between the m1a sub-domain of Otx2 mutants and the developing cerebellum of *Dbx1*^{Cre/+} normal embryos (Fig. 3I,L,O,R,U). In addition, they reveal that, unlike the m1a sub-domain, the m1b sub-domain is unable to activate cerebellar-like expression when Otx2 is ablated at E10; rather, Otx2 appears to be required in this area to promote mesencephalic GABAergic differentiation by controlling post-mitotic transition of GABAergic precursors. At E18.5, the cerebellar expression profile in the m1a sub-domain was even more evident. Indeed, compared with the mesencephalon and the cerebellum of *Dbx1*^{Cre/+} embryos (supplementary material Fig. S3), in Otx2 mutants the combined expression of Pax6, Math1 and Zic1 identified an evident EGL-like structure, the co-expression of Pax2 and Lhx1 presumptive GABAergic interneurons, and the activation of calbindin (Calb) in Lhx1⁺ Pax2⁻ presumptive Purkinje cells (supplementary material Fig. S3).

The m1a sub-domain generates organized cerebellar-like structures

Because *Dbx1*^{Cre/+}; *Otx2*^{flox/flox} mice were viable, fertile and apparently did not show evident behavioral abnormalities, we studied whether post-natally the cerebellar-like cell types generated in the dorsal mesencephalon of Otx2 mutants terminally differentiated into cerebellar-like organized structures. This analysis, performed at post-natal day (P) 7, P20 and P30, clearly indicated that this was the case. Indeed, this conclusion was supported at P7 by (1) the co-expression of Math1, Pax6 and Zic1 in the EGL and co-expression of Pax6 and Zic1 in the IGL (Fig. 4A-D); (2) the co-expression of Calb, parvalbumin (Parv) and Lhx1 in the Purkinje-like cell layer (Fig. 4E-H); (3) the presence of Pax2⁺ Lhx1⁺ neurons corresponding to presumptive GABAergic interneuron precursors in the white matter, or to differentiating Golgi or basket and stellate neurons located in the IGL or the molecular layer, respectively (Fig. 4E,F); and (4) abundant Sox2⁺ GFAP⁺ cells corresponding to differentiating Bergmann glia cells (Sottile et al., 2006) detected in the white matter or migrating towards the Purkinje cell layer (Fig. 4I-L). At P20, the cerebellar-like phenotype was evident along most of the dorsal mesencephalon of *Dbx1*^{Cre/+}; *Otx2*^{flox/flox} mutants

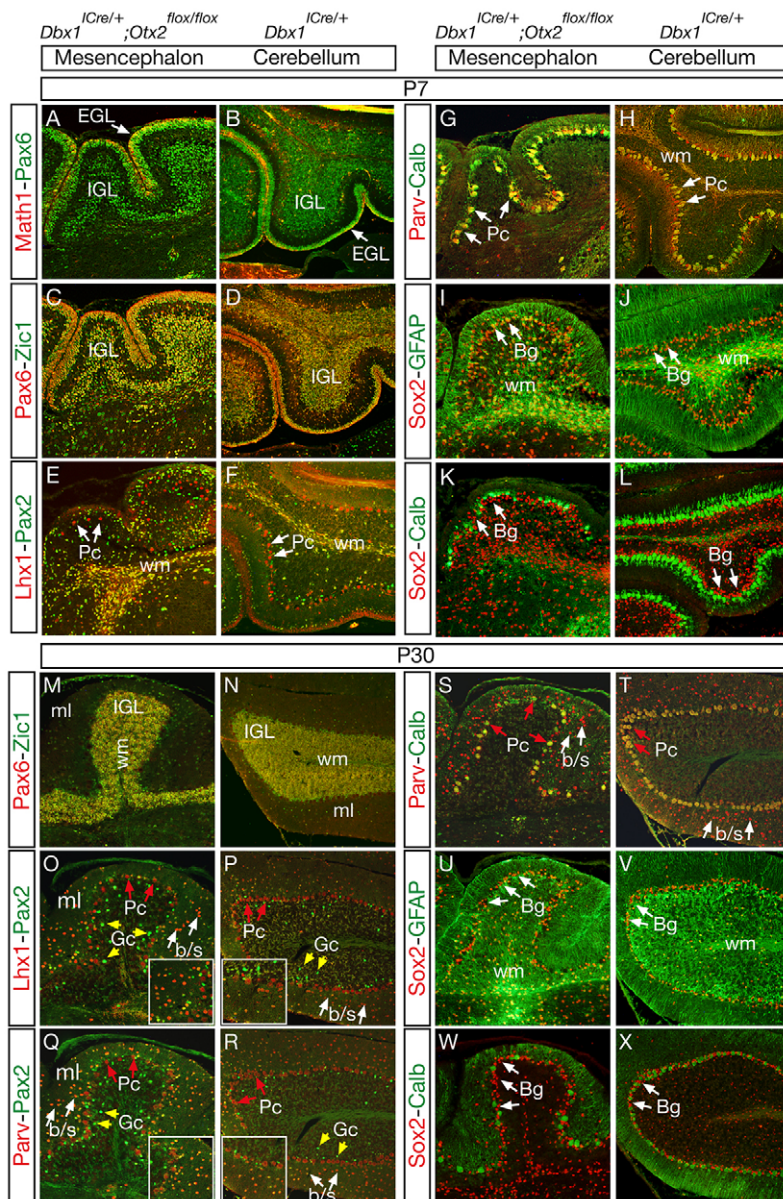


Fig. 4. Cerebellar-like anatomical and histological organization in the dorsal mesencephalon of *Otx2* mutants. Immunohistochemistry experiments performed at P7 (A-L) and P30 (M-X) on the mesencephalon of *Dbx1*^{ICre/+};*Otx2*^{flox/flox} and the cerebellum of *Dbx1*^{ICre/+} with Math1 and Pax6 (A,B), Pax6 and Zic1 (C,D,M,N), Lhx1 and Pax2 (E,F,O,P), Parv and Pax2 (Q,R), Parv and Calb (G,H,S,T), Sox2 and GFAP (I,J,U,V) or Sox2 and Calb (K,L,W,X) show a cerebellar-like structure including the major cerebellar cell-types organized in lobule-like units. At P7, compared with control cerebellum, in *Otx2* mutants Math1 is expressed in the outermost Pax6⁺ sub-compartment of the EGL (A,B, arrows), Pax6 and Zic1 in the developing IGL (C,D), Lhx1, Parv and Calb in Purkinje cells (E-H, arrows), Pax2 and Lhx1 in precursors of GABAergic interneurons (E,F) and Sox2⁺ GFAP⁺ expression in Bergmann glia cells aggregating in proximity of the Purkinje cell layer (I-L, arrows). At P30, the cerebellar tissue of *Otx2* mutants appears fully differentiated and shows Pax6⁺ Zic1⁺ IGL neurons (M,N), Lhx1⁺ Parv⁺ Calb⁺ Purkinje cells (O-T, red arrows), Pax2⁺ Lhx1⁺ Parv⁺ presumptive Golgi cells (O-R, yellow arrowheads), Lhx1⁺ lowPax2⁺ presumptive basket and stellate neurons in the molecular layer (O-T, white arrows) and Sox2⁺ GFAP⁺ Bergmann glia cells intercalated to Purkinje cells (U-X, arrows). The insets in O-R show higher magnification images. Bg, Bergmann glia cells; b/s, basket and stellate interneurons; EGL, external granule cell layer; Gc, Golgi cells; IGL, internal granule cell layer; ml, molecular layer; Pc, Purkinje cells; wm, white matter.

as revealed by morphological inspection of whole brains and Pax6 expression (supplementary material Fig. S4A-H). Importantly, only the posterior-most dorsal mesencephalon did not reveal cerebellar-like histology and Pax6 expression (supplementary material Fig. S4E,H, arrows). At P30, a fully differentiated cerebellar-like identity was evident (Fig. 4M-X). Indeed, as revealed by Pax6 and Zic1, the EGL was extinguished and a mature IGL was detectable (Fig. 4M,N); the molecular layer was populated by Lhx1⁺ low Pax2⁺ Parv⁺ inhibitory interneurons bona fide corresponding to basket and stellate neurons (Fig. 4O-T, white arrows); the IGL included Pax2⁺ Lhx1⁺ Parv⁺ presumptive Golgi neurons (Fig. 4O-R, yellow arrows); and Calb⁺ Parv⁺ Purkinje cells, also expressing Lhx1, were distributed along the external border of the IGL (Fig. 4O-T, red arrows) and were intercalated with Sox2⁺ GFAP⁺ Bergmann glia cells (Fig. 4U-X, white arrows). No abnormalities were detected in cell-type identity and anatomy of the endogenous cerebellum of *Dbx1*^{ICre/+};*Otx2*^{flox/flox} mutants (supplementary material Fig. S4; data not shown). In summary, we have shown that lack of *Otx2* in the dorsal mesencephalon generates severe

abnormalities such that m1a progenitors switch to a coordinated program of cerebellar neurogenesis and m1b progenitors are selectively impaired for post-mitotic transition into GABAergic neurons.

Cerebellar-like cell types originate from dorsal mesencephalic progenitors reprogrammed at specific developmental stages

To investigate the origin of cerebellar-like cell types, we first analyzed whether in *Dbx1*^{ICre/+};*Otx2*^{flox/flox};*R26R* triple mutants, these cell types co-expressed β -Gal and were therefore generated by *Dbx1*⁺ progenitors. Indeed, in this triple mutant, Cre recombinase should both inactivate *Otx2* and constitutively activate *lacZ* mRNA translation in Cre⁺ progenitors and their progeny. At P7, Pax6⁺ EGL-like and differentiating IGL neurons, presumptive Calb⁺ Purkinje cells, Pax2⁺ GABAergic interneurons and Sox2⁺ glial precursors (Fig. 5) all co-expressed β -Gal thus indicating that these cell types were derived from the m1a VZ/SVZ expressing *Dbx1*-driven *ICre* before E10.5. Indeed, *Dbx1* expression was lost after E10.5 in the

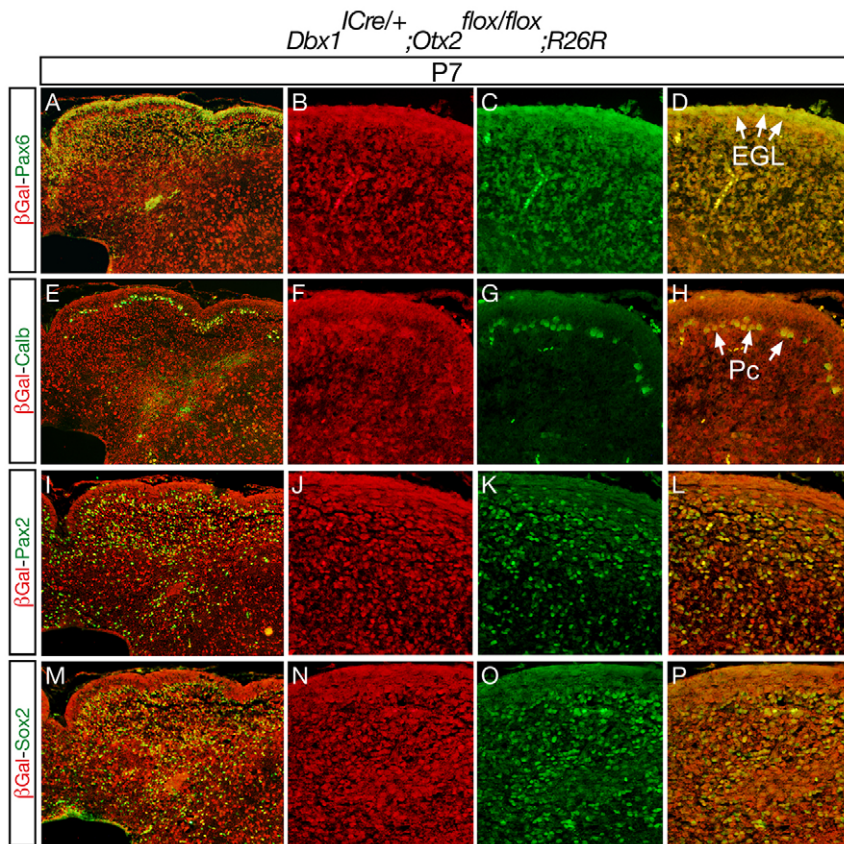


Fig. 5. Genetic cell fate of cerebellar-like cell types generated by *Otx2* mutants. Cell fate analysis performed at P7 on *Dbx1*^{I^{Cre/+}};*Otx2*^{flox/flox};*R26R* mutants shows co-staining between β-Gal⁺ cells and representative cerebellar markers such as Pax6 (A-D), Calb (E-H), Pax2 (I-L) and Sox2 (M-P), thus indicating that all cell types identified in the *Otx2* mutant cerebellar-like structures are generated by progenitors expressing *Dbx1*-driven Ires Cre. EGL, external granule cell layer; Pc, Purkinje cells.</sup></sup>

m1a sub-domain of *Otx2* mutants (Figs 1, 2). This conclusion was also supported by the finding that *Dbx1* was not expressed in the EGL (Fig. 1; supplementary material Fig. S2Q,R; data not shown). Nevertheless, this analysis did not reveal when m1a progenitors were programmed to switch into a specific cerebellar cell fate. To this aim, we first analyzed the neurogenesis of Pax2⁺ and Lhx1⁺ neurons through bromodeoxyuridine (BrdU) short-pulse and birth-dating experiments. BrdU short-pulse experiments performed at E12 and E13.5 showed that virtually no expression was detected for Lhx1 or Pax2 in BrdU⁺ progenitors of both control and *Otx2* mutant embryos (supplementary material Fig. S5A-D), indicating that Lhx1 and Pax2 expression was activated in early post-mitotic progenitors. Then, we studied through birth-dating experiments when proliferating mutant progenitors exited the cell cycle and activated Lhx1 or Pax2 expression. We found that m1a progenitors of control and *Otx2* mutant embryos, labeled with BrdU at E11.5 expressed Lhx1 and Pax2 (only for *Otx2* mutants) at E12.5 (Fig. 6A,B,D,E), and that the number of BrdU⁺ Lhx1⁺ or BrdU⁺ Pax2⁺ neurons was diminished when BrdU was administered at E12.5 and embryos analyzed at E13.5 (Fig. 6J,K,M,N). A similar pattern of neurogenesis was detected for Lhx1⁺ and Pax2⁺ neurons in the normal cerebellum (Fig. 6C,F,L,O). Further experiments based on cumulative BrdU labeling at E11.7, E11.85 and E12 showed that at E15.5 most of the Lhx1⁺ or Pax2⁺ neurons detected in the m1a sub-domain of *Otx2* mutants and in the mesencephalon and cerebellum of control embryos were BrdU⁺ (supplementary material Fig. S5E-J). These findings suggest that compared with the normal cerebellum, the temporal generation of Lhx1⁺ and Pax2⁺ neurons in the m1a sub-domain of *Otx2* mutants and control embryos (only for Lhx1) was not influenced by *Otx2* and occurred primarily between E11.5 and E12.5. By contrast, the generation of Lhx1⁺ neurons in the m1b sub-

domain of *Dbx1*^{I^{Cre/+}};*Otx2*^{flox/flox} mutants was severely impaired (Fig. 6A,B,J,K), whereas that of Pou4f1⁺ post-mitotic neurons was much less affected (Fig. 6G,H,P,Q). To extend the similarity with cerebellar neurogenesis, we assessed whether a fraction of Pax2⁺ cells was mitotically active in the early post-natal cerebellum (Weisheit et al., 2006). BrdU short-pulse experiments at P4 showed that some of the Pax2⁺ cells were also BrdU⁺ in both mutant mesencephalon and control cerebellum (Fig. 6S,T, arrowheads). A further experiment in which BrdU was administered at P4 and Pax2⁺ BrdU⁺ cells were analyzed at P7 confirmed the proliferating activity of Pax2⁺ neuronal precursors (Fig. 6U,V, arrowheads). Then, we analyzed the origin and proliferating activity of presumptive EGL progenitors. The experiments described above showed that EGL-like cells could not be detected before E15 (Fig. 3; data not shown). In particular, four out of six of the E15.5 mutant embryos scored exhibited Pax6 expression in the VZ/SVZ and early EGL (Fig. 3) whereas the residual two embryos exhibited Pax6 expression only in the VZ/SVZ (data not shown). Instead, in E18.5 *Otx2* mutants (*n*=7) the EGL was always detected (supplementary material Fig. S3; data not shown). These findings suggest that Pax6 was activated at around E15 in VZ/SVZ progenitors just before the EGL progenitors migrated towards the outermost mesencephalon. To strengthen this possibility and in addition to genetic cell-fate experiments (Fig. 5), we first performed a birth-dating experiment in which we administered BrdU every three hours from E11.7 up to E12 and from E13.3 up to E13.6 and analyzed respectively at E15.5 and E16.5, whether Pax6⁺ or Math1⁺ cells were also BrdU⁺. We found that in both cases several of the Pax6⁺ or Math1⁺ cells were also BrdU⁺ (Fig. 7B,D,F, arrows). Then, we analyzed whether presumptive EGL cells represented a proliferating population of expanding precursors by performing BrdU pulse experiments and</sup></sup>

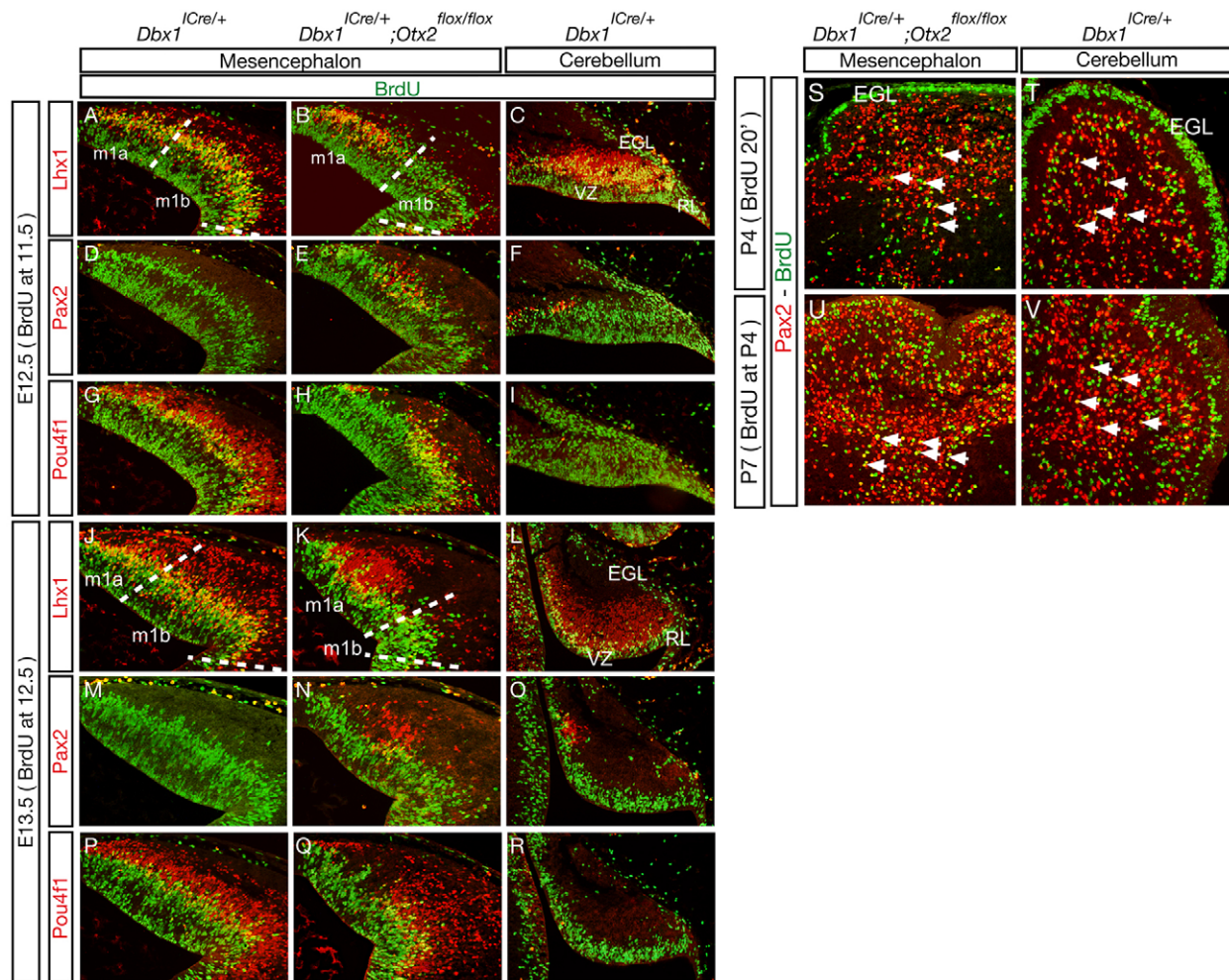


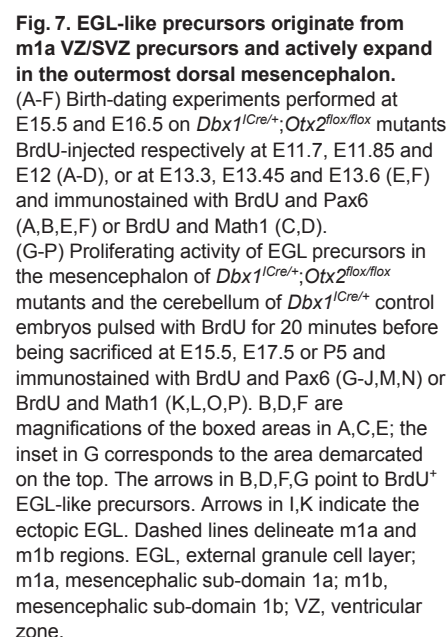
Fig. 6. Progenitors committed to generate Pax2⁺, Lhx1⁺ and Pou4f1⁺ post-mitotic precursors exit the cell cycle primarily between E11.5 and E12.5, but mitotically active Pax2⁺ precursors can be detected at early post-natal stages. (A–R) E12.5 and E13.5 *Dbx1*^{Cre/+} and *Dbx1*^{Cre/+}; *Otx2*^{flox/flox} embryos BrdU-injected respectively at E11.5 and E12.5 are immunostained with BrdU and Lhx1 (A–C, J–L), BrdU and Pax2 (D–F, M–O) or BrdU and Pou4f1 (G–I, P–R). Note that, compared with normal mesencephalon, in *Otx2* mutants BrdU⁺ Lhx1⁺ post-mitotic precursors are remarkably reduced in the m1b sub-domain where, by contrast, BrdU⁺ Pou4f1⁺ neurons are abundantly generated. (S–V) Proliferating activity of Pax2⁺ precursors detected at P4 in the mesencephalon of *Dbx1*^{Cre/+}; *Otx2*^{flox/flox} mutants and the cerebellum of *Dbx1*^{Cre/+} mice sacrificed 20 minutes after a single injection of BrdU (S, T) or analyzed 3 days after a single injection of BrdU (U, V). Several Pax2⁺ BrdU⁺ cells are indicated (arrowheads). EGL, external granule cell layer; m1a, mesencephalic sub-domain 1a; m1b, mesencephalic sub-domain 1b; RL, rhombic lip; VZ, ventricular zone.

found that at E15.5 some of the Pax6⁺ EGL-like cells were actively proliferating (Fig. 7G, arrows), even though, compared with E15.5 control cerebellum, the number of BrdU⁺ Pax6⁺ cells was remarkably lower (Fig. 7H). However, the number of Math1⁺ and Pax6⁺ proliferating cells increased in parallel with the thickness of the EGL, which exhibited no obvious difference from the control cerebella at later stages (Fig. 7I–P). Thus, together with cell-fate experiments (Fig. 5) and marker analysis (Figs 2–4), birth-dating and BrdU pulse experiments suggest that in *Otx2* mutants early EGL founders originate from the m1a VZ/SVZ, migrate at ~E15.5 to the outermost mesencephalon (Fig. 3) and then vigorously expand as a self-sustaining clonal population that subsequently differentiates into IGL neurons (Fig. 4).

Otx2 stage-dependent suppression of cerebellar cell fates

Finally, we studied whether the suppression of the cerebellar cell types by *Otx2* is temporally restricted to specific developmental stages. To this aim, we inactivated *Otx2* using a mouse model carrying tamoxifen (Tx)-inducible CreER under the transcriptional

control of *Otx2* (*Otx2*^{CreER}) (Johansson et al., 2013) and administered Tx during embryonic development such that we could assess the *Otx2* requirement from E8.5 up to E17.5. Mutant embryos receiving Tx at E8.5 and/or E9.5 exhibited at E12.5 a marked anterior expansion of Fgf8 expression (data not shown) and at E18.5 cerebellar expansion at the expense of mesencephalon and were therefore excluded from this analysis. Instead, compared with *Otx2*^{CreER/+} control embryos, those inactivating *Otx2* through sequential Tx administration at E10.5, E11.5 and E12.5 revealed at E13.5 only a slightly expanded expression of Fgf8 in the MHB region (data not shown) and, importantly, exhibited at E18.5 a Pax6⁺ EGL-like structure and numerous Pax2⁺ cells (Fig. 8A,B,D,E,G,H). E18.5 embryos receiving Tx at E11.5, E12.5 and E13.5 showed fewer Pax6⁺ cells stalling in proximity of the midline and only a small EGL-like structure (Fig. 8F, arrows), whereas Pax2⁺ cells remained numerous (Fig. 8I). *Otx2* inactivation from E12.5 up to E15.5 was not sufficient to activate Pax6 expression (Fig. 8O,P), but was sufficient to allow the differentiation of Pax2⁺ cells (Fig. 8S,T), generation of which was, however, not observed when Tx was



(Puelles et al., 2003; Puelles et al., 2004; Prakash et al., 2006; Omodei et al., 2008); and (3) regulates as a post-mitotic selector neuronal subtype identity in the ventral tegmental area (Di Salvio et al., 2010; Di Giovannantonio et al., 2013). Nevertheless, little is known about its cell-autonomous role in progenitors and/or neurons of the dorsal mesencephalon. In this study, *Dbx1*-driven *ICre*, as the endogenous *Dbx1*, is not expressed in the posterior-most mesencephalon close to the MHB, and *Otx2* is not inactivated in this area, which was apparently unaffected. This has allowed us to study for the first time the *Otx2* cell-autonomous role in the dorsal m1 progenitor domain, which generates GABAergic and glutamatergic neurons (Nakatani et al., 2007; Kala et al., 2009). Our data indicate that this domain can be further subdivided into a dorsal m1a *Zic1*⁺ sub-domain and a ventral m1b *Zic1*⁻ sub-domain. We have shown that *Otx2* is uniformly expressed in m1 progenitors whereas post-mitotically it is restricted with *Gata2* and *Lhx1* to the GABAergic cell lineage. *Otx2* inactivation in m1 progenitors has revealed that *Otx2* is differentially required in the m1a and m1b sub-domains. Loss of *Otx2* in the m1a sub-domain generates a dramatic modification in the identity and fate of progenitors, which fail to maintain the expression of *Dbx1* and *Pax7* and, importantly, do not activate the expression of *Hes1* and *Gata2*, two determinants of the mesencephalic GABAergic lineage. Nevertheless, the generation of *Lhx1*⁺ *Gad65*⁺ neurons is not affected but rather increased. To reconcile this apparent contrast, we have reasoned that in the absence of local *Otx2*, m1a progenitors might acquire the identity and fate of a different brain region not requiring *Hes1*

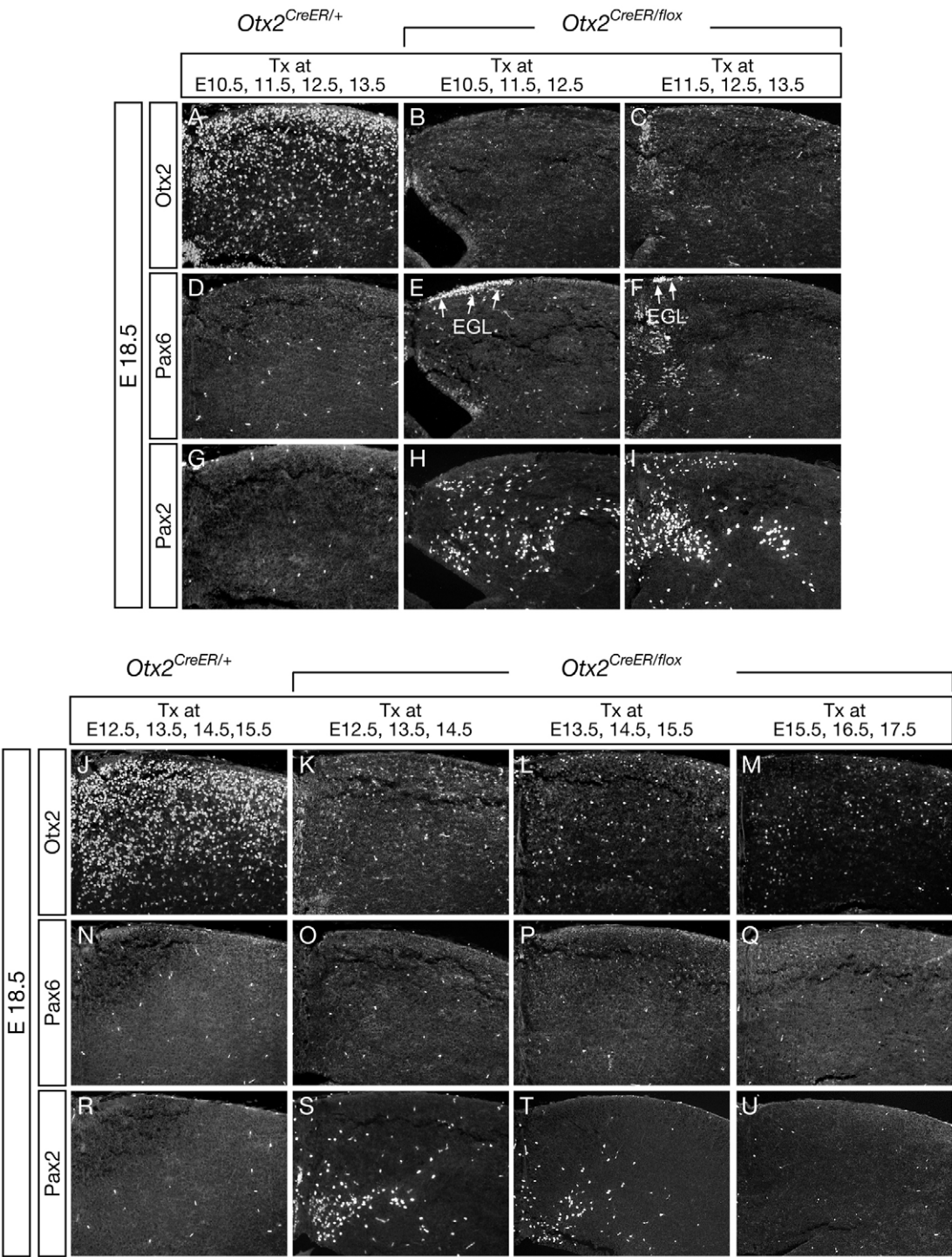


Fig. 8. Temporal competence of dorsal mesencephalon to Otx2-dependent suppression of Pax6⁺ and Pax2⁺ cell generation. Immunohistochemistry experiments with Otx2 (A–C, J–M), Pax6 (D–F, N–Q) or Pax2 (G–I, R–U) on *Otx2*^{CreER/+} and *Otx2*^{CreER/flox} embryos sacrificed at E18.5 and Tx injected at the indicated stages show that in Otx2 mutants the activation of Pax6 occurs when Tx is administered between E10.5 and E11.5 (E,F) whereas Pax2⁺ cells are detected at all stages of Tx administration (H,I,S,T) except when Tx is provided between E15.5 and E17.5 (U). The arrows in E,F point to EGL-like Pax6⁺ precursors. EGL, external granule cell layer.

or Gata2 for GABAergic differentiation. Accordingly, our data indicate that the territorial identity and neurogenesis of the m1a progenitor sub-domain undergo a mesencephalic to cerebellar fate switch (supplementary material Fig. S6A). Indeed, the m1a sub-domain differentiates into cerebellar lobule-like structures showing a high order of anatomical and histological organization very

similar to that identified in a normal cerebellum. These data thus suggest that, at least in the m1a sub-domain, Otx2 is cell-autonomously required either only to suppress cerebellar neurogenesis, or, it might play a dual active role, that is, suppressor of cerebellar fate and activator of the mesencephalic differentiation program. This introduces two important aspects: (1) the relationship

between the MHB organizing activity and the molecular competence of early progenitors in interpreting this activity to induce mesencephalic differentiation; and (2) the *Otx2* role as a permissive factor or an instructive determinant for mesencephalic differentiation. On the basis of this and previous reports (Puelles et al., 2004; Acampora et al., 1997; Millet et al., 1999; Martinez-Barbera et al., 2001; Liu and Joyner, 2001b; Foucher et al., 2006; Heimbucher et al., 2007; Agoston and Schulte, 2009), these aspects can now be better discussed. Data from the present study suggest that, in an embryonic context where MHB integrity is retained, *Otx2* may cell-autonomously impart anterior polarity to MHB-inducing signals. This leads us to argue that the cerebellar fate should represent a basic program induced in the absence of *Otx2* on both sides of the neuroectoderm flanking the MHB organizer as indeed occurs in *Dbx1^{lCre/+};Otx2^{fllox/fllox}*.

We and others have shown that *Otx2* may directly interact with the *Grg4* (*Tle4*) co-repressor in cell culture experiments and embryos (Puelles et al., 2004; Heimbucher et al., 2007), suggesting that through this interaction *Otx2* may act as a repressor in early steps of mesencephalic specification. More recently, a relevant study identified *Meis2* as a second *Otx2*-interacting partner, in this case with co-activating property. The authors proposed that *Meis2* controls dorsal mesencephalic development through direct interaction with *Otx2* and without the MHB organizer contribution (Agoston and Schulte, 2009). In addition, *Grg4* and *Meis2* are sequentially expressed during embryonic development, *Grg4* being expressed at a higher level than *Meis2* at early stages and vice versa at later stages, thus suggesting that at later stages *Meis2* may successfully compete with *Grg4* for the binding to *Otx2* (Agoston and Schulte, 2009). In our study, *Meis2* and *Grg4* expression were not remarkably affected in *Dbx1^{lCre/+};Otx2^{fllox/fllox}* embryos between E11 and E14 (supplementary material Fig. S7). Therefore, together with previous studies, our data lead us to propose that *Otx2* is sequentially required during dorsal mesencephalic development, early on as a repressor of cerebellar fate by interacting with *Grg4* and subsequently as an activator of mesencephalic neurogenesis by interacting with *Meis2*. According to this proposal: (1) in the dorsal mesencephalon of chick embryos, loss of *Meis2* in an '*Otx2* normal' context does not result in cerebellar differentiation but rather in abnormal mesencephalic development (Agoston and Schulte, 2009), suggesting that *Otx2* without *Meis2* is sufficient to suppress cerebellar neurogenesis; and (2) in the absence of *Otx2*, the *m1a* sub-domain undergoes a mesencephalic to cerebellar fate switch, suggesting that *Grg4* and *Meis2* without *Otx2* could not suppress cerebellar fate or promote dorsal mesencephalic differentiation (this study). Importantly, these phenotypes occur independently of the MHB organizing activity. We also showed that the dorsal mesencephalic territory able to differentiate into cerebellum is restricted only to the *m1a* *Zic1⁺* sub-domain. This might reflect an *m1a*-specific gene expression code and/or a regionally restricted temporal competence to the cerebellar suppression by *Otx2*. We showed that this territory exhibits a different temporal permissiveness to the generation of *Pax6⁺* EGL progenitors and *Pax2⁺* interneurons. Indeed, the generation of *Pax6⁺* EGL progenitors occurs when *Otx2* is ablated between E10 and E11.5, and that of *Pax2⁺* interneurons when *Otx2* is lost between E10 and E14.5 (supplementary material Fig. S6A). Thus, it remains to be assessed whether *Otx2* ablation earlier than E10 in embryos retaining a normal positioning and activity of the MHB organizer results in a wider mesencephalic conversion into cerebellar fate. Finally, this study also reveals that lack of *Otx2* in the *m1b* sub-domain generates a remarkably different phenotype. Indeed, the *m1b*

progenitors retain a fairly normal identity but fail to generate most of the *Gata2⁺* early post-mitotic precursors and the large majority of the *Lhx1⁺* *Pax7⁺* GABAergic neurons; by contrast, the generation of *Pou4f1⁺* glutamatergic neurons is much less affected in this sub-domain. Thus, according to the restricted expression in GABAergic post-mitotic neurons, *Otx2* may represent a novel intrinsic factor required in the *m1b* sub-domain for post-mitotic transition and/or terminal differentiation of *Helt⁺* GABAergic progenitors (supplementary material Fig. S6B). This suggests that *Otx2* may function downstream of *Mash1* and *Helt* and upstream of *Gata2* and *Lhx1*.

MATERIALS AND METHODS

Mouse mutants

The *Dbx1^{lCre/+}*, *Otx2^{CreER/+}* and *Otx2^{fllox/+}* mouse strains have been previously reported (Johansson et al., 2013; Bielle et al., 2005; Causseret et al., 2011; Puelles et al., 2003).

In situ hybridization and immunohistochemistry

In situ hybridization experiments were performed as described (Simeone, 1999) with *Cre*, *Dbx1*, *5'Otx2*, *Fgf8*, *Gbx2*, *Pax2* and *Wnt1* probes each corresponding to a PCR fragment of variable length between 300 and 700 base pairs (Puelles et al., 2003; Puelles et al., 2004). Immunohistochemistry experiments were performed as previously reported (Omodei et al., 2008). Antibodies raised in rabbit were against *Otx2* (1:3000; gift of G. Corte, CBA, Genoa, Italy), *Dbx1* (1:75; A. Pierani), *Zic1* (1:400; Novus Biologicals, NB600-488), *Gata2* (1:250; Santa Cruz Biotechnology, sc9008), *Helt* (1:200; kindly provided by Y. Ono, KAN Research Institute, Kobe, Japan), *Lhx1* (1:300; Abcam, ab14554), *Pax2* (1:400; Zymed, 71-6000), *Math1* (1:100; kindly provided by J. Johnson, UT Southwestern Medical Center, Dallas, USA), *Sox2* (1:500; Chemicon, AB5603), *vGlut2* (1:75; Synaptic Systems, 135403), *Gad65* (1:2500; Sigma, G5163), *calretinin* (1:400; Swant, 7699/4) and *Grg4* (1:250; Santa Cruz Biotechnology, sc9125); antibodies raised in mouse were against *Pou4f1* (1:100; Santa Cruz Biotechnology, sc8429), *Pax7* (1:100; Developmental Studies Hybridoma Bank, supernatant), *Mash1* (1:60; BD Pharmingen, 556604), *Lhx1* (1:150; Developmental Studies Hybridoma Bank, cl. 4F2), *Pax6* (1:100; Developmental Studies Hybridoma Bank, concentrated), *calbindin* (1:100; Swant, CB300), *parvalbumin* (1:300; Sigma, P3088), *GFAP* (1:200; Chemicon, MAB3402) and *Meis2* (1:150; Abnova, H00004212-H01); antibodies raised in goat were against *Otx2* (1:100; R&D Systems, AF1979), *Ngn1* (1:50; Santa Cruz Biotechnology, sc19231), *calbindin* (1:100; R&D Systems, AF3320), *Sox2* (1:400; R&D Systems, AF2018), *Lhx1* (C20) (1:100; Santa Cruz Biotechnology, sc19341); antibodies against β -Gal (1:1500; Abcam, ab9361) and 5'-bromo-2'-deoxyuridine (BrdU) (1:1500; Novus Biologicals, NB500-169) were raised in chicken and rat, respectively.

BrdU labeling experiments and Tx administration

BrdU pulse experiments were performed by administering pregnant females or post-natal mice with a single injection of BrdU at 50 mg/kg body weight. Embryos or post-natal mice were sacrificed 20 minutes later. For cell-fate experiments, BrdU was administered to pregnant females in a single or maximum three cumulative injections (every 3 hours) and mutants were collected one or more days later or, as in some experiments, were sacrificed post-natally. For experiments involving conditional inactivation of *Otx2* by *Otx2*-driven *CreER*, Tx was administered by three sequential (one per day) intraperitoneal injections at 50 mg/kg body weight for each injection.

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Competing interests

The authors declare no competing financial interests.

Author contributions

L.G.D.G. and M.D.S. performed most of the analysis of *Dbx1^{Cre/+};Otx2^{fllox/fllox}* mutants; D.O. analyzed the *Otx2^{CreER/flox}* mutants; N.P. and W.W. contributed to data interpretation; A.P. provided the *Dbx1^{Cre/+}* mutant, the Dbx1 antibody and contributed to data interpretation; D.A. analyzed the data and contributed to write the manuscript; A.S. conceived the experiments, interpreted most of the data and wrote the manuscript.

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Supplementary material

Supplementary material available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.102954/-DC1

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