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 isthmic 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 (Cossley 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 Grog4) 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

RESEARCH ARTICLE
Otx2 cell-autonomously determines dorsal mesencephalon versus cerebellum fate independently of isthmic organizing activity
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(Neurolg1 – 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 (Dbx1ICre), which inactivates Otx2 in progenitors of the m1 domain without affecting MHB integrity (Bielle et al., 2005; Causseret 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,I), 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.S1L,L’,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 Dbx1ICre/+;Otx2floxflox 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 Dbx1ICre/+;Otx2floxflox control embryos, in Dbx1ICre/+;Otx2floxflox 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 Dbx1ICre/+;Otx2floxflox 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 Dbx1ICre/+;Otx2floxflox 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 Dbx1ICre/+;Otx2floxflox 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 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-
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/subventricular 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, 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 cerebellar fate switch may occur in the m1a sub-domain. We found that, like in the cerebellum, in the dorsal mesencephalon...
Fig. 2. Loss of Otx2 remarkably affects identity and differentiation of m1 progenitors. Immunohistochemistry experiments performed at E11, E12.5 and E13.5 on Dbx1ICre/+;Otx2flox/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.

Dbx1ICre/+;Otx2flox/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 Dbx1Cre+ and Dbx1Cre+;Otx2flox/+ 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 Dbx1Cre+;Otx2flox/+ mutants Lhx1 is expressed prevalently in the m1a sub-domain where a fraction of Lhx1+ cells co-express 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 Dbx1Cre+ and on the mesencephalon of Dbx1Cre+;Otx2flox/+ 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 (G), 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 Dbx1Cre+ 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 Dbx1Cre+ normal 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 Dbx1Cre+;Otx2flox/+ 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 Dbx1Cre+;Otx2flox/+ mutants.
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 arrowheads); 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 Dbx1ICre+/−;Otx2flox/flox;R26R 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 Dbx1ICre+/−;Otx2flox/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, 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 and Parv 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+ Parv- 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.
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 Dbx1ICre/+;Otx2fl/fl;R26R 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

![Fig. 5. Genetic cell fate of cerebellar-like cell types generated by Otx2 mutants. Cell fate analysis performed at P7 on Dbx1ICre/+;Otx2fl/fl;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.](image-url)
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 (Otx2CreER) (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 Otx2CreER/+ 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
provided from E15.5 onwards (Fig. 8U). Thus, these data indicate a differential sensitivity to the Otx2-dependent suppression of the EGL-like (Pax6+) and cerebellar GABAergic interneuron (Pax2+) phenotypes. Indeed, suppression of Pax6 expression and EGL-like fate requires Otx2 up to ~E11.5, whereas Pax2 suppression requires Otx2 up to E14.5.

DISCUSSION

The molecular mechanism that controls regional identity and subsequent neurogenesis in the mesencephalon is a matter of intense study for the relevance of neuronal cell types generated by this territory, such as dopaminergic, GABAergic and glutamatergic neurons and their subtypes (Wurst and Bally-Cuif, 2001; Liu and Joyner, 2001a; Smidt and Burbach, 2007; Nakatani et al., 2007; Kala et al., 2009; Andersson et al., 2006; Omodei et al., 2008; Di Salvio et al., 2010). These studies showed that extrinsic signals produced by organizing centers are initially required to instruct competent progenitors to activate intrinsic pathways of differentiation determining maturation and post-mitotic transition of progenitors into immature and, subsequently, terminally differentiated neurons. During mesencephalic development, Otx2 is required at multiple levels in these processes. In particular, Otx2 (1) controls the specification of the whole territory by positioning the ISO at the MHB (Acampora et al., 1997; Broccoli et al., 1999; Martinez-Barbera et al., 2001; Puelles et al., 2003; Millet et al., 1999); (2) is intrinsically required to control proliferation and differentiation of dopaminergic and red nucleus progenitors (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 Helt 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 Helt...
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^CreER^; Otx2^flox/flox^.

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^CreER^; Otx2^flox/flox^ 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 subdomain 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 GATABergic 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^CreER^, Otx2^CreER^ and Otx2^flox^ mouse strains have been previously reported (Johansson et al., 2013; Bielle et al., 2005; Causeret 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 Gr4 (1:250; Santa Cruz Biotechnology, sc9125); antibodies raised in mouse were against Pou4f1 (1:100; Santa Cruz Biotechnology, sc4429), 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 Grx2 (1:1000; R&D Systems, AF1979), Ngn1 (1:50; Santa Cruz Biotechnology, sc19341); antibodies against β-Gal (1:1500; Abcam, ab9361) and 5′-bromo2′-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.


