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Temporal and spatial delineation of mouse *Otx2* functions by conditional self-knockout

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To identify the independent spatial and temporal activities of the essential developmental gene *Otx2*, the germline mutation of which is lethal at embryonic day 8.5, we floxed one allele and substituted the other with an inducible *CreER* recombinase gene. This makes '*trans*' self-knockout possible at any developmental stage. The transient action of tamoxifen pulses allows time-course mutation. We demonstrate efficient temporal knockout and demarcate spatio-temporal windows in which *Otx2* controls the head, brain structures and body development.

Keywords: CreER^{T2}; tamoxifen; development; brain; *Otx2*
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INTRODUCTION

Although regulatory genes frequently control several developmental steps (Kmita & Duboule, 2003), most loss-of-function studies demonstrate only their earliest roles (Nagy *et al*, 2003). The successive functions of a given gene and the length of its activity have been inaccessible until recently. In mice, the CreER recombinase system is a promising tool for addressing these questions (Santagati *et al*, 2005). To disclose the many functions and the corresponding time-windows of an essential gene, *Otx2*, we have added a twist to CreER-based conditional knockout, to allow spatially and temporally controlled gene invalidation during short developmental periods. The *Otx2* homeobox gene has key roles in early antero-posterior patterning (Kimura-Yoshida *et al*, 2005), neuroectoderm induction and maintenance (Rhinn *et al*, 1998), and rostral brain and cranio-facial development (Acampora *et al*, 1995; Matsuo *et al*, 1995; Ang *et al*, 1996). Conditional knockout studies using constitutive Cre mice have shown that *Otx2* is required around mid-gestation for neuronal specification in the retina (Nishida *et al*, 2003) and in the ventral midbrain (Puelles *et al*, 2004; Vernay *et al*, 2005). To further explore the significance of its persistent expression in many brain structures (Frantz *et al*, 1994; Mallamaci *et al*, 1996), we performed time-course self-knockout between embryonic day (E)10.5 and E18.5, delineating the requirement of *Otx2* during the later stages of development.

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RESULTS

Strategy

Temporally and spatially controlled ablation of the *Otx2* gene can be achieved using alleles that are complementarily modified (Fig 1A): a pre-mutant *flox* allele carrying *loxP* sites flanking the essential *Otx2* exon 2 and a recombinase-expressing *CreER* allele in which the tamoxifen-inducible CreER^{T2} encoding gene (Feil *et al*, 1997) replaces the Otx2 coding region (for the mouse lines, see supplementary Figs S1,S2 online). Breeding an *Otx2*$^{flox/CreERT2}$ male with an *Otx2*$^{flox/flox}$ female yields 50% heterozygous *Otx2*$^{flox/CreERT2}$ embryos that are prone to conditional invalidation and 50% *Otx2*$^{flox/flox}$ control embryos. A single pulse of tamoxifen triggers *Otx2* invalidation in 6–48 h (Hayashi & McMahon, 2002) only in cells expressing *Otx2* at that time. Importantly, the target gene is preserved in all other territories (Fig 1B), which enables one to discriminate between activities in independent domains. Strict spatial control necessitates that the recombinase be produced throughout the *Otx2* expression domains. A comparison of the *Otx2* and *CreER*^{T2} pattern in *Otx2*$^{+/CreERT2}$ mice showed perfect superimposition at all stages (Fig 1C).

Efficient *Otx2* deletion at all stages

We first asked whether a single tamoxifen injection could temporarily block the function of *Otx2*. To test this, we attempted to reproduce defects in its known early functions. We triggered mutagenesis at E7.5, expecting a head phenotype as found in hypomorphs or chimaeras (Matsuo *et al*, 1995; Rhinn *et al*, 1998). All tamoxifen-injected *Otx2*$^{flox/CreERT2}$ embryos (23 out of 23) showed abnormal head development (Fig 2A), whereas *Otx2*$^{flox/flox}$ animals (20 out of 20) and solvent-injected embryos of both genotypes were normal. From E9.5 onwards, *Otx2*$^{flox/CreERT2}$ embryos showed an absence or reduction of the forebrain, the eye and the inferior mandible, indicating a marked reduction in the dosage of Otx2 (Matsuo *et al*, 1995). Genotype-specific tamoxifen-induced deletion of *Otx2* exon 2 was confirmed by PCR (Fig 2B; for oligos and probes, see supplementary Fig S1 online). The affected areas corresponded precisely to sites that were fate mapped by their *CreER*^{T2} expression at E7.5, as shown

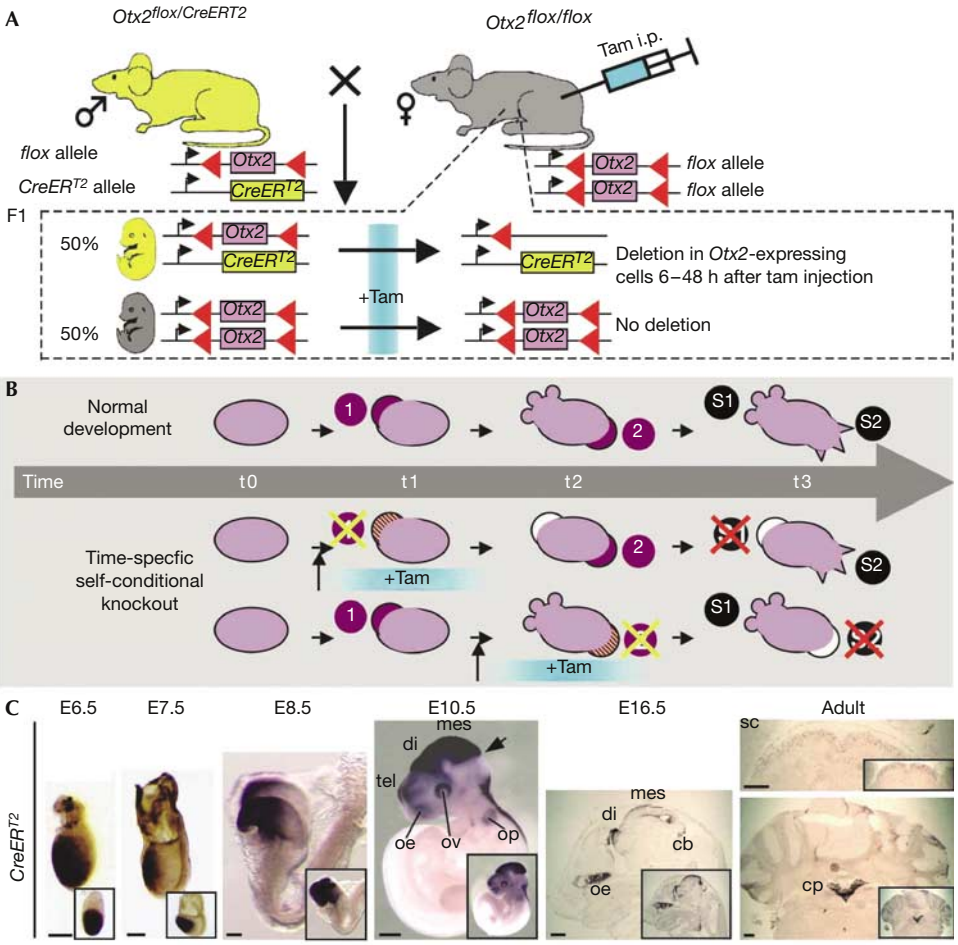

Fig 1 | The knock-in-based *CreER*^{T2} strategy. (**A**) The mating of an *Otx2*$^{flox/CreERT2}$ male with a homozygous *Otx2*$^{flox/flox}$ female yields 50% *Otx2*$^{flox/CreERT2}$ and 50% *Otx2*$^{flox/flox}$ embryos. Intraperitoneal tamoxifen injection (Tam i.p., blue) triggers gene deletion only in *Otx2*$^{flox/CreERT2}$ embryos and solely in cells expressing *Otx2*. Purple and yellow boxes correspond to *Otx2* and *CreER*^{T2} genes, respectively, and red triangles to *loxP* sites. (**B**) Potentialities of the method. During normal development (top), two spatially and temporally distinct *Otx2* expression sites (purple circles 1 and 2) control the development of structures S1 and S2. Depending on when CreER^{T2} is activated (+tam, yellow stripes), the *Otx2* gene is deleted in region 1 (middle) or 2 (bottom), perturbing development of structure S1 or S2, respectively. (**C**) *In situ* hybridization of *Otx2*$^{+/CreERT2}$ embryos at embryonic day (E)6.5–E10.5 (whole mount) and E16.5 (sagittal sections) and adults (transversal sections) at the level of mesencephalon (top) and cerebellum (bottom) with *CreER*^{T2} and *Otx2* probes (insets; for probes used, see supplementary Figs S1,S2 online). The arrow points to the isthmus. Scale bars, 100 μm for E6.5–E8.5, 500 μm for E10.5, E.16.5 and adult. cb, cerebellum; cp, choroid plexus; di, diencephalon; mes, mesencephalon; oe, olfactory epithelium; op, otic placode; ov, optic vesicle; sc, superior colliculi; tel, telencephalon.

by the *Z/AP* reporter line (Lobe *et al*, 1999), which expresses alkaline phosphatase after Cre-mediated recombination (Fig 2C). Thus, a single early tamoxifen injection can induce typical *Otx2* loss-of-function phenotypes. To study the late functions of Otx2, we then asked whether single injections at later stages yielded similar deletion efficiency. As tamoxifen exerts its effect in 48 h, we performed single injections at E10.5, E12.5, E14.5 and E16.5. Delayed PCR analysis confirmed efficient deletion of the floxed allele at all stages tested (Fig 2D). In addition, *in situ* analysis of *Otx2* messenger RNA with exon 2 probe 30 h after injection showed a marked decrease in the signal, demonstrating the rapidity of tamoxifen action under all conditions (Fig 2E–L). Finally, ectopic onset of *Math1* gene expression was found in the mesencephalon 30 h after injection (see supplementary Fig S3I,M online). As it is normally restricted to the more posterior regions (Fig 4J), this confirmed that Otx2 protein activity is quickly abolished. Thus, we could achieve *Otx2* inactivation at its endogenous sites of expression at precise stages of development.

Specific time windows for late activities of *Otx2*

To study *Otx2* functions during the later stages of development, we used the same injection intervals as above. When injected at E10.5, only 5% of *Otx2*$^{flox/CreERT2}$ animals reached postnatal day 10 (P10), although their proportion was normal at E18.5 (Fig 3A). This shows that around E10.5, *Otx2* controls a developmental step that is necessary for postnatal survival. By contrast, viability was normal and animals were present at the expected ratio (Acampora

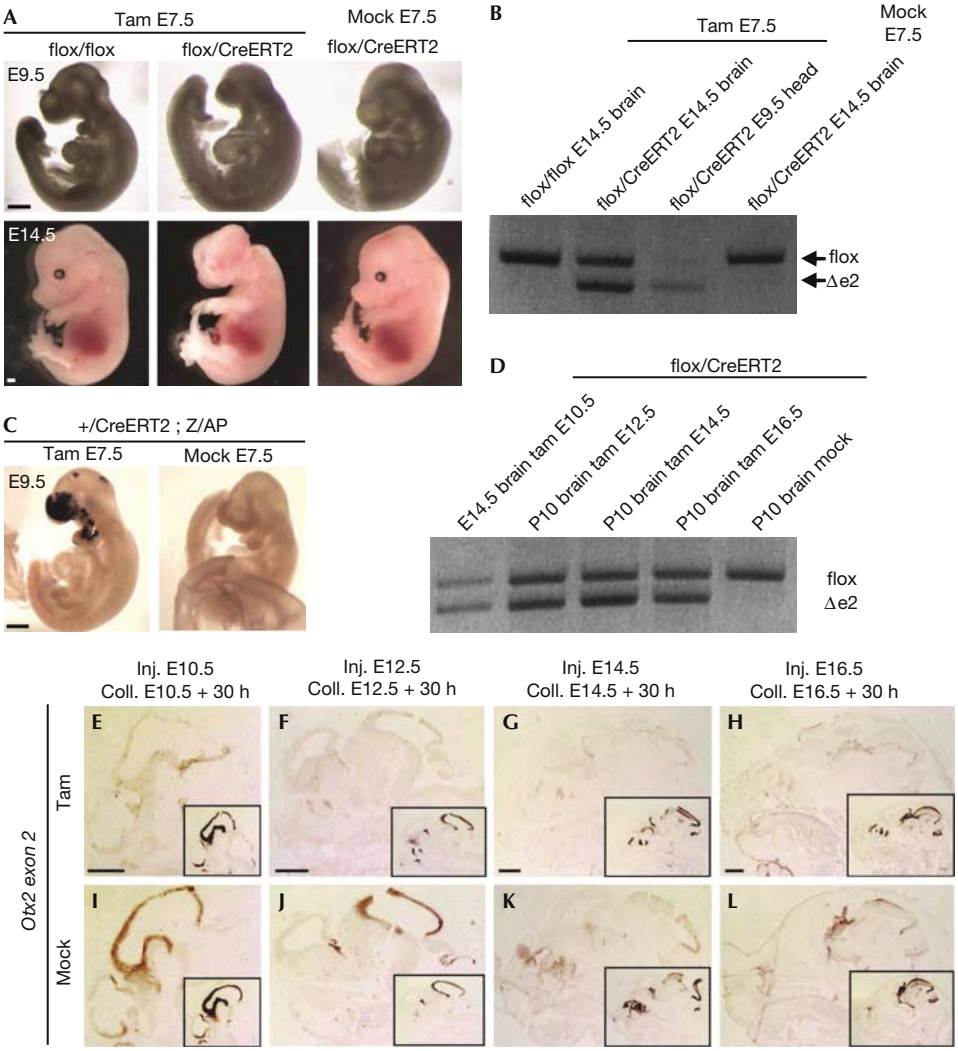

Fig 2 | Efficient *Otx2* deletion after single tamoxifen injection. (**A**) Morphology of animals of indicated age and genotype after tamoxifen (Tam) or solvent (Mock) injection at embryonic day (E)7.5. Scale bars, 500 μm. (**B**) PCR detection of *flox* and deleted exon 2 (Δ*e2*) alleles (for primers, see supplementary Fig S1 online) using DNA from the brain or head of embryos of indicated age and genotype. (**C**) Alkaline phosphatase activity in E9.5 *Otx2*$^{+/CreERT2}$; *Z/AP* embryos that received tamoxifen or solvent at E7.5. Scale bar, 500 μm. (**D**) PCR analysis, as in (**B**), using DNA from the indicated part, and age of *Otx2*$^{flox/CreERT2}$ embryos after tamoxifen (Tam) or solvent (mock) injection at the indicated stages. (**E–L**) *In situ* hybridization with *Otx2* exon 2 probe and *CreER*^{T2} probe (insets; for probes, see supplementary Figs S1,S2 online) of sagittal sections of *Otx2*$^{flox/CreERT2}$ embryos that received tamoxifen (Tam) or solvent (Mock) at indicated times and were collected 30 h later. Scale bars, 1 mm.

et al, 1995) when injection was performed at E12.5 or later. Thus, *Otx2* is crucial for postnatal survival around E10.5 and its requirement ends at E12.5. Similarly, we could mark out the temporal limit of the requirement of *Otx2* for cranial development. Contrary to earlier inactivation, tamoxifen injection at E10.5 and later resulted in morphologically normal embryos (Fig 3B). This implies that *Otx2* acts before E10.5 to determine cranial morphogenesis. We also found that *Otx2*$^{flox/CreERT2}$ animals injected at E12.5, but not E14.5 and E16.5 mutants, were consistently smaller than their *Otx2*$^{flox/flox}$ littermates at 10 weeks (Fig 3C). These animals were born with a normal weight, but then accumulated up to 25% growth retardation (Fig 3D). This indicates the contribution of the *Otx2* gene between E12.5 and E14.5 to postnatal growth.

Examination of the brain of P10 animals injected at E12.5 and E14.5 showed mesencephalon abnormalities, with early injections causing the strongest phenotypes. A large medial portion of inferior colliculi was missing in animals injected at E12.5 (Fig 3E). In animals injected at E14.5, inferior colliculi showed improved development but still did not join medially; however, they seemed to be normal in animals injected at E16.5 (Fig 3F–H).

Sagittal sections showed sequential roles of *Otx2* in midbrain/hindbrain development. In animals injected at E12.5, a posterior medial part of the dorsal midbrain adopted a cerebellar histology, with formation of granular cell layers (Fig 3I). In adults, this ectopic structure showed true cerebellar organization with Purkinje cells, interneurons and internal granular cells, as shown

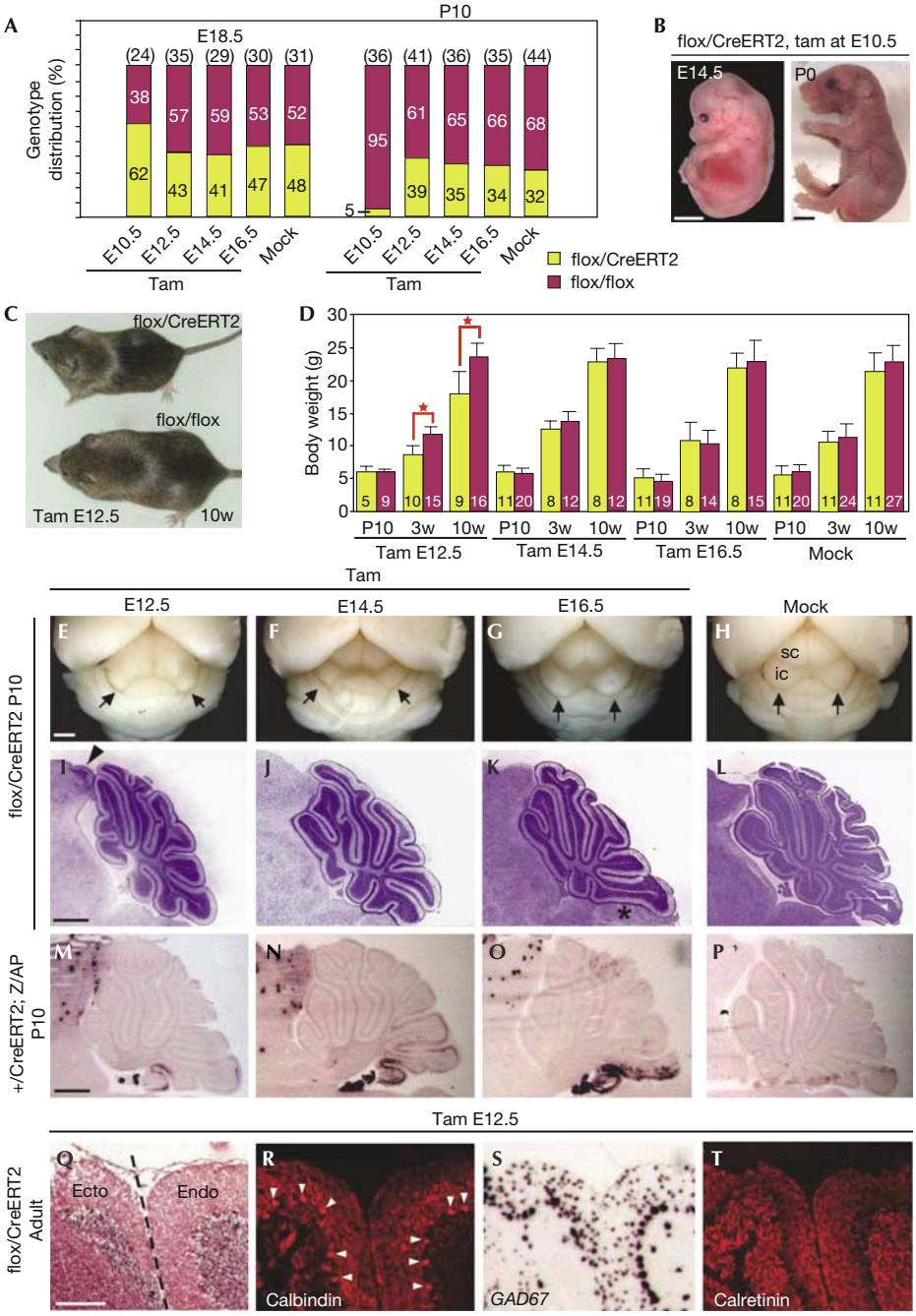

Fig 3 | Biological consequences of *Otx2* invalidation at embryonic day (E)10.5, E12.5, E14.5 or E16.5. (**A**) Genotype distribution, at E18.5 and P10, of F1 animals (Fig 1A) that received tamoxifen (Tam) at the indicated time or solvent (Mock). The number of individuals analysed is indicated at the top of the bars. (**B**) Morphology of E14.5 and P0 $Otx2^{flox/CreERT2}$ mice after tamoxifen injection at E10.5. Scale bars, 2 mm. (**C**) Size comparison of 10-week littermates of the indicated genotypes that received tamoxifen at E12.5. (**D**) Weight distribution of animals of the indicated genotypes at P10, 3 weeks and 10 weeks after birth versus time of tamoxifen (Tam) or solvent (Mock) injection. Histograms represent mean value and error bars are standard deviation. The number of animals in each series is shown at the bottom. *$P<0.001$ after Student's *t*-test. (**E–L**) Anatomical and histological comparison of midbrain/hindbrain development at P10. Dorsal view of the brain (**E–H**) and Nissl-stained sagittal sections (**I–L**) of animals that received tamoxifen at E12.5 (**E,I**), E14.5 (**F,J**), E16.5 (**G,K**) or solvent (**H,L**). Arrows point to the inferior colliculi. The arrowhead points to the granular-like staining of the mesencephalon. Asterisk shows size and foliation defects of posterior cerebellum. ic, inferior colliculus; sc, superior colliculus. (**M–P**) Alkaline phosphatase activity in sagittal sections of brains of P10 $Otx2^{+/CreERT2}$; *Z/AP* animals that received tamoxifen at time corresponding to the panels above. Scale bars, 2 mm. (**Q–T**) Histological organization of ectopic cerebellar-like structure (Ecto) and endogenous cerebellum (Endo) of an adult $Otx2^{flox/CreERT2}$ animal that received a single injection of tamoxifen (Tam) at E12.5. Haematoxylin–eosin staining (**Q**), calbindin immunofluorescence (white arrowheads) (**R**), *GAD67 in situ* hybridization (**S**) and calretinin immunofluorescence (**T**) on sagittal sections. Scale bar, 500 μm.

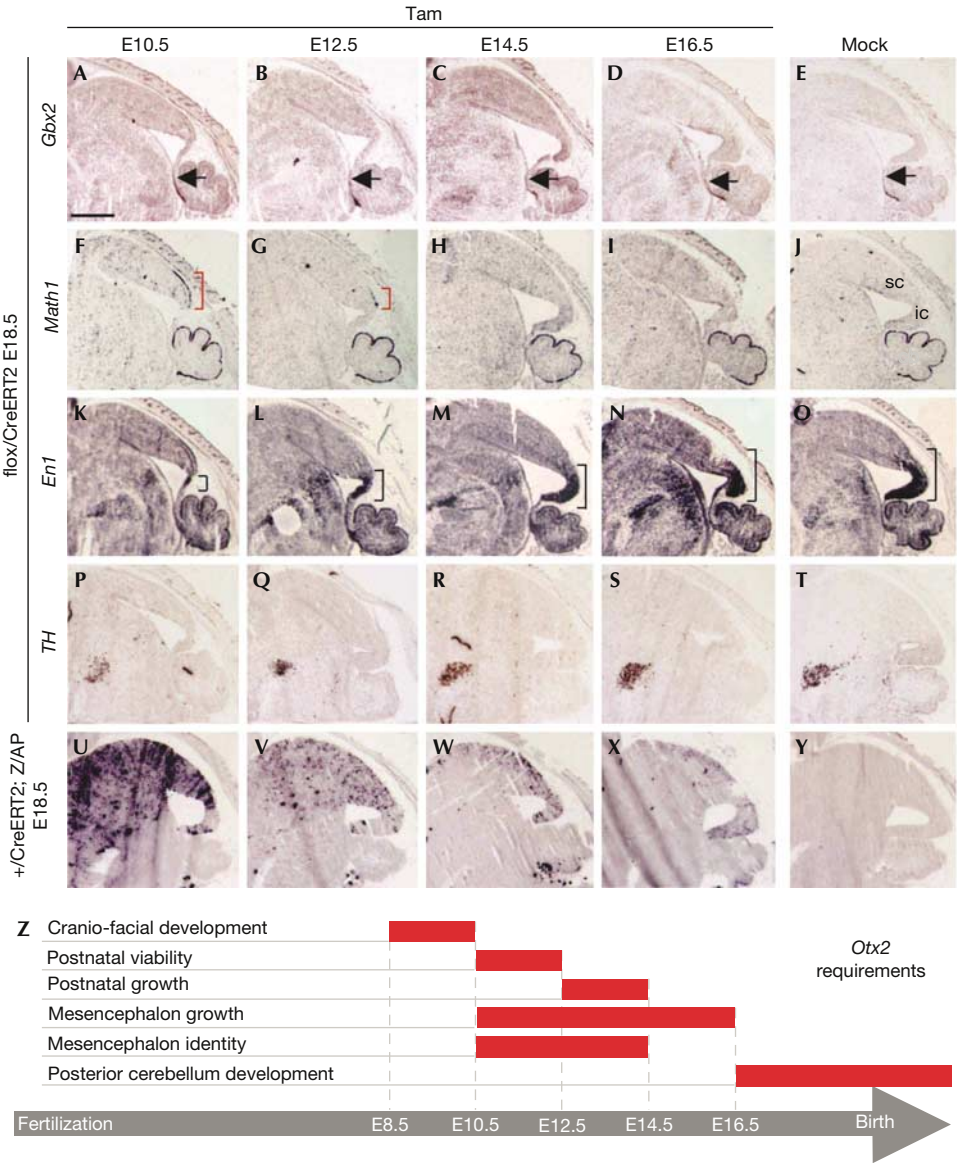

Fig 4 | Midbrain specification under *Otx2* temporal control. (**A–T**) *In situ* hybridization of sagittal sections of embryonic day (E)18.5 brains from *Otx2*$^{flox/CreERT2}$ embryos that received tamoxifen (Tam) at indicated times or solvent (Mock) with *Gbx2* (**A–E**), *Math1* (**F–J**), *En1* (**K–O**) and *TH* (**P–T**). Rostral limit of *Gbx2* expression is marked by black arrows. Ectopic *Math1* expression is marked by red brackets. *En1* labelling extension is indicated by black brackets. cb, cerebellum; ic, inferior colliculus; sc, superior colliculus. (**U–Y**) Alkaline phosphatase activity in sagittal sections of E18.5 *Otx2*$^{+/CreERT2}$; *Z/AP* animals that received tamoxifen at time corresponding to the panels above. Scale bar, 1 mm. (**Z**) Schematic representation of the temporal and spatial delineation (red bars) of the multiple requirements of *Otx2* during the later stages of embryonic development.

by calbindin, *GAD67* and calretinin expression (Fig 3Q–T). In animals injected at E16.5, the mesencephalon and anterior cerebellum seemed to be normal, but a marked reduction of posterior cerebellum development and foliation was observed (Fig 3K). This chronology of *Otx2*-controlled steps corresponds to dynamic changes of the *Otx2* expression areas. Analysis of the progeny of *Otx2*-expressing cells with the *Z/AP* mouse line shows that, as development proceeds, a decreasing number of cells express *Otx2* in the mesencephalon, whereas an increasing number of posterior cerebellum founders start to express *Otx2* (Figs 3M–P,4U–Y).

The different functions and their temporal limits identified in this time-course experiment are summarized in Fig 4Z. We analysed the midbrain changes in greater detail, whereas other studies might focus on the postnatal survival, growth defect and cerebellum phenotypes.

Temporal control of mesencephalon patterning

To study temporal activities of *Otx2* in the mesencephalon of all mutants, their brains were analysed at E18.5. This avoided the perinatal death of embryos injected at E10.5 and allowed complete tamoxifen action in embryos injected at E16.5.

As early midbrain *Otx2* and hindbrain *Gbx2* expression controls the positioning of the isthmus (Martinez-Barbera *et al*, 2001), we checked its location in our mutants. At E18.5, this seemed to be normal in all cases, as shown by *Gbx2* expression (Fig 4A–E). Detection of isthmus markers *Gbx2* and *En1* 30 h and 48 h after E10.5 injection confirmed that the midbrain–hindbrain border was unchanged (supplementary Fig S3A–H online). In embryos injected at E10.5 and E12.5, cerebellum markers *Math1* (Fig 4F–J) and *Pax6* (not shown) labelled a dorsal area anterior to the isthmus. Hence, this ectopic cerebellum resulted from a local change in mesencephalon identity and not from a rostral shift of the hindbrain. Ectopic *Math1* expression was already evident 30 h after injection (supplementary Fig S3I,M online). In embryos injected at E12.5, rostral extension of the labelled area was reduced compared with the E10.5 mutant, whereas its caudal limit was identical in both cases. Immediately posterior was the caudal midbrain, which expresses *En1* and gives rise to inferior colliculi (Wurst *et al*, 1994). Interestingly, this domain never expressed *Math1*. However, it was also affected by the loss of *Otx2* activity: its size was markedly reduced in embryos injected at E10.5 and progressively recovered to normal in embryos injected at E12.5, E14.5 and E16.5 (Fig 4K–O), perfectly correlating with the inferior colliculi phenotype in adults (Fig 3E–H). To explain this defect, we studied cell proliferation and apoptosis in embryos injected at E10.5. Two days after tamoxifen injection, KI-67 antibody showed a strong decrease in proliferation throughout the dorsal mesencephalon (supplementary Fig S3O,P online), whereas cleaved caspase 3 detection showed no change in the rate of apoptosis (not shown). This differently affected the parts of the midbrain, because, at this stage, superior colliculi have nearly reached their definitive size, whereas inferior colliculi are still to develop. Consistently, we observed thinner superior colliculi and reduced inferior colliculi (Fig 4K).

In the ventral midbrain, Vernay *et al* (2005) have shown that *Otx2* ablation before E12.5 generally results in a decreased number of dopaminergic neurons. We analysed this population in our mutants using *tyrosine hydroxylase* (Fig 4P–T) and *Lmx1b* (not shown) markers. The dopaminergic population seemed to be reduced in embryos injected at E10.5 and E12.5 but was normal elsewhere. In agreement with Vernay *et al* (2005), we found no gross difference in cell proliferation (supplementary Fig S3O,P online) and apoptosis in the ventral midbrain. This indicates that *Otx2* is required up to E14.5 to allow full differentiation of this neuronal population. Fate mapping of midbrain *Otx2*-expressing cells (Fig 4U–Y) showed that E14.5 is the time at which *Otx2*-expressing precursors no longer contribute to the ventral midbrain.

Together, these results discriminate between different functions of *Otx2* in the mesencephalon: it is necessary up to E14.5 to prevent a dorsal area fated to form superior colliculi from adopting a cerebellar fate and to allow differentiation of ventral dopaminergic neurons; it is necessary up to E16.5 to ensure normal growth of the caudal midbrain (Fig 4Z).

DISCUSSION

Here, we report the use of a knock-in *CreER^{T2}* allele over a *floxed* allele combined to transient CreER^{T2} activation by single tamoxifen pulses to capture temporal gene activity. The strategy has several advantages. First, it accurately delivers Cre enzyme to the sites of expression of the gene of interest. Second, it can be used during as broad a developmental period as the gene of interest is expressed. Third, contrary to ubiquitously expressed *CreER* genes, which, once activated, lead to thorough deletion of the floxed gene, the knock-in *CreER^{T2}* gene only disrupts the floxed gene in cells in which it is expressed at the time of tamoxifen injection. This spares other areas in which later gene expression will occur.

We focused on *Otx2* phenotypes between E10.5 and E18.5. Efficient conditional knockout was proved by the reproduction of loss-of-function phenotypes and by the consistency of defects after injection at each stage. Previously, it was shown that *Otx2* is required for cranio-facial development (Matsuo *et al*, 1995). Conditional knockout studies have shown the need for *Otx2* around E10.5–E12.5 for correct patterning of the mesencephalon along the antero-posterior and dorso-ventral axes (Puelles *et al*, 2004; Vernay *et al*, 2005). The study by Vernay *et al* (2005) showed that beyond E12.5, although both patterns are established, *Otx2* is still necessary to maintain mesencephalon identity and allow development of the posterior cerebellum. Using our allelic combination, we could, in a single time-course experiment, reproduce these findings and uncover new specific requirements of *Otx2* for inferior colliculi development, survival at birth and postnatal body growth. Furthermore, we marked out temporal limits beyond which *Otx2* is no longer necessary for developmental activities. Our data show that the requirement of *Otx2* for cranio-facial development ends at E10.5. Similarly, we demarcate temporal windows for *Otx2* control of survival after birth (E10.5–E12.5), body growth (E12.5–E14.5), superior colliculi identity (E10.5–E14.5), ventral midbrain neuronal differentiation (E10.5–E14.5), caudal mesencephalon development (E10.5–E16.5) and posterior cerebellum development (E16.5 and onwards). Cross-checking this information with *Otx2* expression pattern should help in identifying the developing structures that are affected by the mutation.

In the chick, FGF8 signalling from the isthmic organizer induces a cerebellar fate, and this is counteracted in the mesencephalon by *Otx2* expression (Sato *et al*, 2001). A recent study suggests that in zebrafish, the primary function of FGF8 is to keep Otx proteins, which are potent repressors of cerebellar fate, out of the rostral hindbrain (Foucher *et al*, 2006). The transformation of superior colliculi into cerebellum, which we found after E10.5 *Otx2* ablation, supports similar mechanisms in the mouse. In addition, the temporal reduction of transformed area shows anterior to posterior progressive stabilization of superior colliculi identity. Strikingly, the most caudal region of the mesencephalon, which gives rise to inferior colliculi, does not undergo cerebellum transformation, although it requires *Otx2* activity up to E16.5 to develop to its normal size. This shows different *Otx2* activities in subdivisions of the caudal mesencephalon. The existence of such subsegments has recently been documented in the chick embryo (Hidalgo-Sanchez *et al*, 2005). Finally, both inferior colliculi and posterior cerebellum defects point to a potential role for *Otx2* in the control of proliferation. This is in line with the *Otx2* overexpression found in human medulloblastomas, which are aggressive tumours arising from cerebellar granular cell precursors (Di *et al*, 2005).

METHODS

Mouse production and tamoxifen administration. Animals were maintained in the 129/Sv background (for details, see the supplementary information online). Day of vaginal plug was taken as E0.5. Tamoxifen (Sigma-Aldrich, St Louis, MO, USA), diluted at 10 mg/ml in sunflower oil, was administrated at 1 mg per 20 g of body weight. The protocols were approved by the French CGG and CREA committees.

Analyses of embryos and brains. For whole-mount analyses, samples were dissected in PBS and fixed in 4% paraformaldehyde (PFA) for *in situ* analyses, or in 0.2% glutaraldehyde for AP staining. For analyses of the sections, samples were dissected in PBS, fixed in 4% PFA, dehydrated and embedded in paraffin for Nissl staining, or protected in 30% sucrose and frozen in cryomount for haematoxylin–eosin staining and immunofluorescence, or directly frozen on solid CO_2 for alkaline phosphatase and *in situ* studies. Sections (5–12 μm) were processed and Nissl and haematoxylin–eosin staining was carried out according to standard protocol. Alkaline phosphatase staining and whole-mount *in situ* hybridization were carried out as described by Wilkinson (1992) and Lobe *et al* (1999). Probes for *in situ* hybridization were as follows: 0.15 kb *Otx2* exon 2, Cre coding sequence (see supplementary Figs S1,S2 online); *Otx2*, *Math1*, *Gbx2*, *En1*, *GAD67* and *TH* probes were gifts from S. Vincent, B. Vernay, G. Martin, A. Joyner and M. Wassef. Immunofluorescence was performed as described by Vernay *et al* (2005). Primary antibodies used were 1:5,000 rabbit anti-calbindin D-28k (Swant, Bellinzona, Switzerland) and 1:5,000 rabbit anti-calretinin (Chemicon, CA, USA).

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

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