Research article

Segmental identity and cerebellar granule cell induction in rhombomere 1

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Abstract

Background: Cerebellar granule cell precursors are specifically generated within the hindbrain segment, rhombomere 1, which is bounded rostrally by the midbrain/hindbrain isthmus and caudally by the boundary of the *Hoxa2* expression domain. While graded signals from the isthmus have a demonstrable patterning role within this region, the significance of segmental identity for neuronal specification within rhombomere 1 is unexplored. We examined the response of granule cell precursors to the overexpression of *Hoxa2*, which normally determines patterns of development specific to the hindbrain. How much does the development of the cerebellum, a midbrain/hindbrain structure, reflect its neuromeric origin as a hindbrain segment?

Results: We show that a *Gbx2*-positive, *Otx2*-/*Hoxa2*-negative territory corresponding to rhombomere 1 forms prior to an identifiable isthmic organiser. Early global overexpression of *Hoxa2* at embryonic day 0 has no effect on the expression of isthmic signalling molecules or the allocation of rhombomere 1 territory, but selectively results in the loss of granule cell markers at embryonic day 6 and the depletion of cell bodies from the external granule cell layer. By comparison the trochlear nucleus and locus coeruleus form normally in ventral rhombomere 1 under these conditions. Microsurgery, coupled with electroporation, to target *Hoxa2* overexpression to rhombic lip precursors, reveals a profound, autonomous respecification of migration. Rhombic lip derivatives, normally destined to occupy the external granule cell layer, violate the cerebellar boundary to form a ventrolateral nucleus in a position comparable to that occupied by rhombic lip derived neurons in rhombomere 2.

Conclusions: Different overexpression strategies reveal that the recognition of migration cues by granule cell precursors is dependent on their identity as rhombomere 1 derivatives. Segmental patterning cues operate autonomously within the rhombic lip precursor pool. By contrast, a subset of coextensive nuclei is refractory to ectopic *Hoxa2* and is presumably induced solely by isthmic organiser activity. Thus, graded (isthmic) and segmental mechanisms may operate exclusively of one another in the specification of different neuronal populations within rhombomere 1. The early designation of an *Otx2*-negative, *Hoxa2*-negative region, prior to the appearance of the isthmic organiser, is a key initial step in the specification of the cerebellum.

Background
Subdivision along the rostrocaudal axis of the developing central nervous system assigns regional identity to neuronal precursor pools, influencing the fate of their progeny and generating structural diversity [1]. Strategies for conveying fine-grain positional information vary along the length of the axis. For example, the hindbrain is a transiently rhombomeric structure organised by segmentally expressed *Hox* genes. By contrast, the development of the midbrain and forebrain, which lie within the *Otx* expression domain, is regulated by signalling centres generating graded patterning cues. At the transition point, where the hindbrain system of segmentation gives way to regional patterning by local gradients in the midbrain, the most anterior hindbrain segment, rhombomere 1 (r1), expresses neither *Hox* or *Otx* genes and displays aspects of both patterning systems. A number of distinct neuronal populations are generated in r1, including cerebellar granule cells [2], the most populous neuron in the vertebrate CNS. How graded and segmental cues interact in specifying r1 precursors is key to understanding the origins of the cerebellum, a major structure in the higher vertebrate brain.

A number of studies have examined different facets of r1 specification. The rostral boundary of r1 comprises the midbrain/hindbrain isthmus, an important signalling centre in the development of the midbrain and r1. Loss of isthmic signalling severely disrupts both midbrain and cerebellar development [3-5], while application of FGF8, the putative isthmic signalling molecule, suggests a direct involvement of the isthmic organiser in cerebellar induction [6,7]. Moreover, the trochlear motor nucleus and the locus coeruleus, the latter of which is rostrocaudally coextensive with granule cell production, are born within r1 and are induced by isthmic FGF [5,8,9].

In addition to its isthmic identity, r1 also displays properties of a hindbrain segment. Rhombomeres 1 to 3 are defined by the expression of *Gbx2*: within this domain, r1 differs from the more caudal segments by the absence of *Hox* gene expression. In mice, a null mutation in *Hoxa2*, the only *Hox* gene expressed in adjacent r2, results in the caudal expansion of the cerebellum [10] and rostralisation of the second branchial arch to a first arch identity [11,12]. This suggests, in the absence of any changes in isthmic signalling, that suppressing *Hox* gene expression in the hindbrain is sufficient to confer an r1 identity, as experimentally confirmed in zebrafish [13]. Correspondingly, in chick, overexpression of *Hoxa2* in r1 induces ectopic r2-like motor neurons [14], while in the first branchial arch, neural crest adopts second branchial arch characteristics [15,16]. These apparent transformations support a model that r1, as r2, is a homeotic iteration within the meristic series that comprises the rhombencephalon.

To examine isthmic and segmental cues in cerebellar development, we have used a combination of retroviral and microsurgical techniques to assess patterning in r1, in particular with respect to the production of granule cell precursors from the rhombic lip. This population undergoes two distinct phases of migration, the first giving rise to the external granule cell layer (EGL), which covers the pial surface of the cerebellum, and a second in which granule cells migrate radially inwards to form the granule cell layer. We find that while nuclei whose induction is dependent on isthmic signalling remain unchanged, the formation of the external granule cell layer is inhibited by ectopic *Hoxa2*. This suggests that both segmental and isthmic-derived patterning mechanisms may operate independently within r1. Furthermore, microsurgically targeted misexpression of *Hoxa2* in the rhombic lip reveals that the loss of granule cell from the cerebellum can be explained by a dramatic re-specification of tangential migration. A complementary grafting strategy demonstrates that these changes in the patterning of migration occur autonomously at the rhombic lip.

Results
Overexpression of Hoxa2 does not affect r1 allocation or isthmic identity
Previous fate-maps have demonstrated that at embryonic day (E) 2 (stage [st.]10), r1 is defined by the boundaries of the expression domains of, rostrally, *Otx2* [17] and, caudally, *Hoxa2* [2]. By such criteria, *in situ* hybridisation establishes that a *Gbx2*-positive territory corresponding to rhombomere 1 is apparent from the earliest onset of *Otx2* and *Hoxa2* expression (Fig. 1A). By st.9-, the caudal boundary of *Otx2* expression defines the midbrain/hindbrain boundary, or isthmus (mhb; Fig. 1B), an important signalling centre that expresses a number of molecules involved in patterning adjacent territory, including *FGF8*, *Engrailed* and *Wnt1* [4].

Given that the isthmus has an important role in patterning the midbrain and maintaining r1 identity, we first assessed the effect of global *Hoxa2* overexpression on key isthmic genes. The RCASBP(B)*Hoxa2* retrovirus was injected into E0 chick eggs. The expression of *FGF8* (Fig. 1C), *Engrailed (en)1* (Fig. 1D) at E3 and *Wnt1* at E6 (data not shown) were examined at the midbrain/hindbrain boundary. The gross morphology of the neural tube in these embryos was normal and r1 territory formed appropriately. Isthmic genes were expressed in domains encompassing the isthmus that were indistinguishable from controls (injected with RCANBP(B)*Hoxa2*; data not shown). This suggests that the signalling properties of the isthmus are unaffected by ectopic *Hoxa2* at the isthmus.
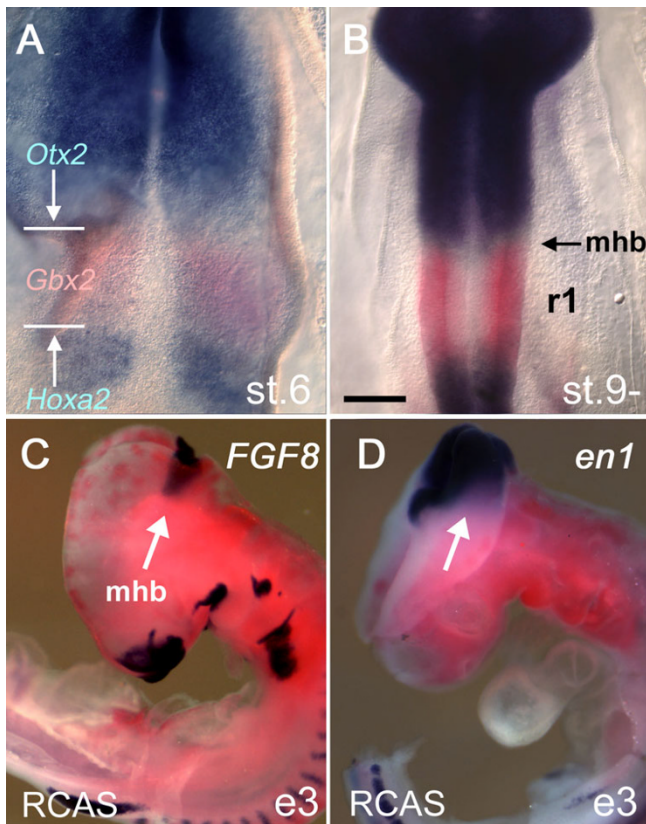
Figure 1
Establishment of rhombomere 1: Isthmic gene expression is refractory to *Hoxa2* overexpression (A) At stage [st.] 6, *Otx2* and *Hoxa2* expression (both in blue) are separated by a *Gbx2*-positve domain (red) that defines rhombomere 1 (r1). The domain of *Gbx2* expression encompasses r1–3. **(B)** At st.9⁻, the boundaries of the *Otx2* and *Hoxa2* expression domains define the segmental boundaries of r1 as determined by fate-map experiments [2, 17]. **(C)** Viral overexpression of RCASBP(B)*Hoxa2* (red) at embryonic day (E) 0 fails to suppress the activation of either *FGF8* (blue) or **(D)** *en1* at E3 (e3). Scale bar (A,B) = 200 μm. mhb – midbrain/hindbrain isthmus.

Having determined that isthmic function was likely to be unimpaired, we chose to examine the effect of ectopic *Hoxa2* on the development of three definitive r1 neuronal populations: cerebellar granule cell precursors, trochlear motor neurons and the locus coeruleus. Of these, only the origin of cerebellar granule cells has been microsurgically fate-mapped to r1 in chick [2]. Trochlear motor neurons and the catecholaminergic interneurons of the locus coeruleus can be allocated to r1 by the expression of their associated characteristic neuronal markers in relation to the molecular r1 boundaries defined above.

Hoxa2 *overexpression in r1 leads to a cell-depleted EGL*
RCASBP(B)*Hoxa2* retrovirus was injected into the neural tube of st.10 (E2) embryos and neuronal phenotype assessed by *in situ* hybridisation, as flatmounts at E6 (Fig. 2A) or in transverse section at E9. At both ages, widespread infection with RCASBP(B)*Hoxa2* led to a variable reduction in the size of the cerebellum, which is derived from dorsal r1 and becomes distinguishable at E5.

Cerebellar granule cell precursors are generated at the rhombic lip (E6 onwards), which comprises the interface between the neural tube and non-neural roofplate of the fourth ventricle. During their characteristic ventrolateral tangential migration to form the EGL, granule cell precursors express a number of distinct molecular markers, including a paired-homeodomain transcription factor, *Pax6* [18], and the cell surface receptor, *erbB4* [19]. At E6, *Pax6* labels cells condensing as the EGL in dorsal r1 (Fig. 2B). This dorsal expression is lost following *Hoxa2* overexpression (Fig. 2C), along with a small domain of ventricular *Pax6* expression (Figs 2B,2C, white arrows), just anterior to the normal limit of *Hoxa2* expression (Fig. 2B, inset). Loss of dorsal *Pax6* suggests that rhombic lip derivatives either fail to be generated or that they down-regulate *Pax6* during their tangential migration. At E9, the EGL is strongly positive for *erbB4*, as illustrated in an embryo infected with the control virus RCANBP(B)*Hoxa2* (Fig. 2D). Following overexpression of *Hoxa2*, *erbB4* becomes restricted to small islands of superficial cerebellum (Fig. 2E, arrows) overlying a locally thickened cerebellum. As with *Pax6*, no ectopic expression of *erbB4* was observed that might account for an apparent loss of granule cells. The depletion of this cell type was confirmed by cresyl violet staining of control RCAN- (Fig. 2F) and RCAS-infected (Fig. 2G) cerebellum. Strikingly, *Hoxa2* overexpression at E2 results in a cerebellum displaying a clearly demarcated external cerebellar layer at E9 that is nevertheless devoid of cell bodies.

Hoxa2 *overexpression does not affect trochlear nucleus or locus coeruleus formation*
The structure of the trochlear nucleus and locus coeruleus was assessed by the expression of *Phox2a* (Fig. 3A), a characteristic marker of both neuronal pools within r1 [20]. The locus coeruleus is generated dorsal to the trochlear motor nucleus (Fig. 3B iv), which lies just caudal to isthmus and the oculomotor nucleus (Fig. 3B iii) of the midbrain (Fig. 3B). In transverse section the locus coeruleus can be seen to condense adjacent to the ventricular layer in ventral r1, while dorsally *Phox2a* expression is excluded from the presumptive cerebellum (Fig. 3C). Within the hindbrain, caudal of the r1/2 boundary, *Phox2a* expression is organised into contiguous lateralised columns (Fig. 3A). Global overexpression of *Hoxa2* has no effect on the size or position of either the trochlear motor nucleus
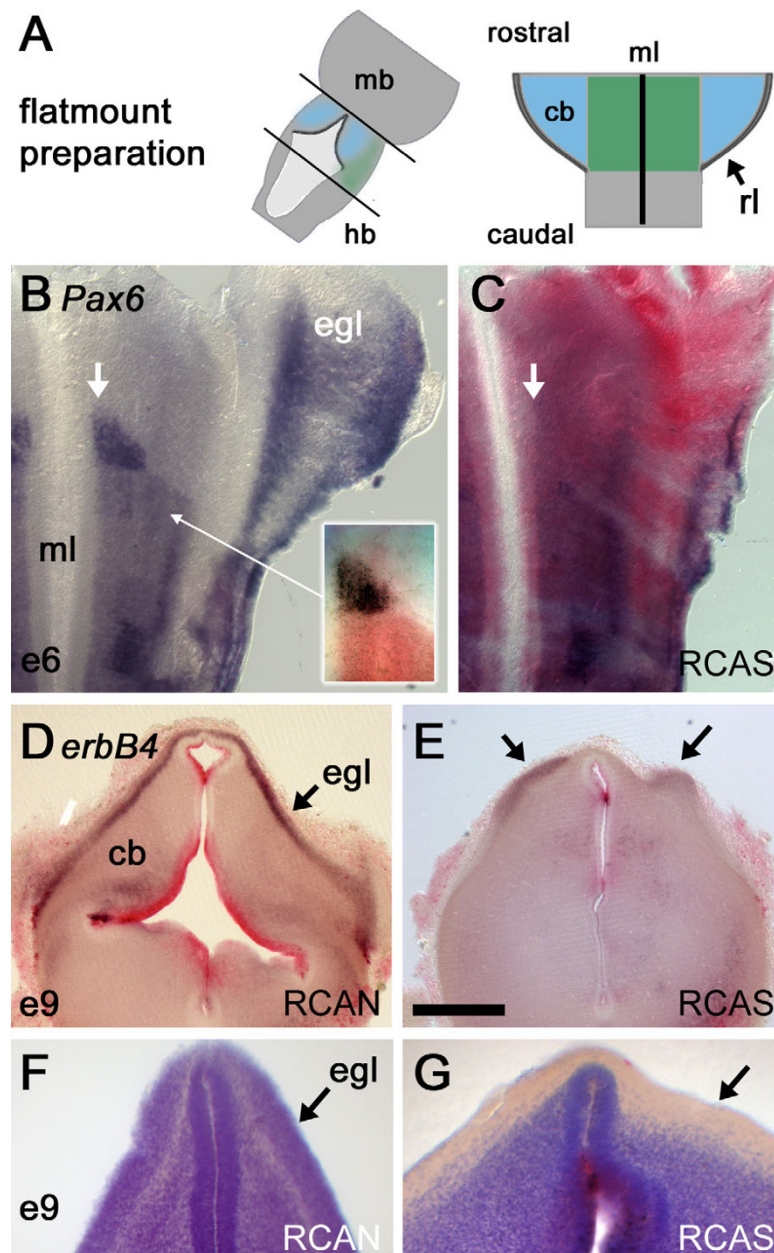
Figure 2
Global overexpression of *Hoxa2* depletes rhombomere 1 of granule cell precursors (A) Flatmounts of brains up to embryonic day (E) 6 were prepared by opening the neural tube along the dorsal midline: cb – cerebellum, mb – midbrain, hb – hindbrain, rl – rhombic lip, ml – ventral midline. **(B)** The expression of *Pax6* (blue) at E6 in one half of the anterior hindbrain viewed as a flatmount (rostral top, mediolateral axis runs left to right). Granule cell precursors born at the rhombic lip express *Pax6* as they migrate over dorsal rhombomere 1 (r1) to condense as the external granule cell layer (EGL [egl]). *Pax6* is also expressed in a ventral wedge of r1 precursors (arrow) abutting the anterior r2 boundary and ventral midline. The relationship of this *Pax6* positive precursor pool (blue) to the anterior boundary of *Hoxa2* (red) is shown inset. **(C)** Overexpression of *Hoxa2* (red) leads to a loss of *Pax6* in the EGL and prominent ventral wedge (arrow). This is replaced by a weaker, broadened, ventral *Pax6* domain similar to that in the hindbrain. **(D)** At E9, in transverse section through a control embryo, the neuregulin receptor *erbB4* labels the EGL. Red label dorsally reflects high levels of expression of the control virus, RCANBP(B)*Hoxa2* (red), within the ventricular zone of the cerebellum (cb). **(E)** Injection of the active RCASBP(B)*Hoxa2* virus leads to a reduction in *erbB4*. Patches of expression (arrows) correspond to residual EGL overlying a locally thickened cerebellum. **(F)** Cresyl violet stains in control and **(G)** RCASBP(B)*Hoxa2*-infected cerebella reveal an absence of cell bodies within a superficial, subpial layer (arrows), normally occupied by the EGL, following *Hoxa2* overexpression. Scale bar (D,E) = 500 μm.

or the locus coeruleus (Fig. 3D). Curiously, the level of *Phox2a* transcripts appears higher in trochlear motor axons following viral infection.

By contrast, in dorsal r1, *Phox2a* expression was induced throughout the presumptive cerebellum excluding the rhombic lip (Fig. 3D, asterisk). In transverse section, ectopic *Phox2a* expression in the cerebellum lies within the mantle, outside the cell-depleted EGL (Fig. 3E, asterisk). Dorsal r1 is the origin of ventrally migrating locus coeruleus neuroblasts, raising the possibility that *Hoxa2* may have generated an ectopic locus coeruleus population within dorsal r1. However, *in situ* hybridisation for the enzyme tyrosine hydroxylase (TH), which is expressed at the earliest stages of locus coeruleus migration [21], demonstrates that ectopic *Phox2a*-positive cells are not catecholaminergic: in RCAS infected embryos, TH is expressed only within the ventral pool of locus coeruleus neurons (Fig. 3F).

Hoxa2 overexpression disrupts the migration of rhombic lip derivatives in r1

The loss of granule cell markers and up-regulation of *Phox2a* in deeper cerebellar layers raise the possibility that the migration and/or fate of rhombic lip derivatives are specifically affected by ectopic *Hoxa2*. Correspondingly, a previous lineage study hypothesised that the locus coeruleus is derived from the EGL [22], which might favour an argument that *Hoxa2* transfection biases production of rhombic lip derivatives towards a deeper-migrating, *Phox2a*-positive derivative.

To assess the fate and movement of r1 rhombic lip derivatives expressing *Hoxa2*, we devised a microsurgical chimaera strategy whereby gene overexpression could be targeted precisely to the rhombic lip precursor pool [23]. RCASBP(B)*Hoxa2* plasmid was electroporated into donor quail tissue at E2. Dorsal r1, which maps to the cerebellar rhombic lip in later embryos [2,23], was then transplanted unilaterally into dorsal r1 of a host chick embryo at E2. At E6, the fate of quail cells derived from the rhombic lip was logged using a quail specific perinuclear marker, Q¢PN, and double *in situ* hybridisation for both *Hoxa2* and *Phox2a* (Fig. 4A).

Figure 4B shows a wholemount cerebellum from a control (uninfected) quail–chick chimaera at E6. Darkly stained graft-derived precursors occupy the narrow insertion of roofplate at the midline of the cerebellum. Where quail cells overlap the rhombic lip (more caudally), migrating EGL precursors are distributed across the surface of the cerebellum (black arrow). In Fig. 4C, the distribution of rhombic lip derivatives is profoundly changed when quail tissue electroporated with *Hoxa2* is used as donor tissue (*n* = 6/7). No quail cells are seen in the presumptive cerebel-

lum (dorsal r1). Instead, rhombic lip derivatives expressing high levels of *Hoxa2* condense in ventrolateral r1 (white arrow).

The effect of exclusively targeting *Hoxa2* to the rhombic lip is seen more clearly in transverse section. In control chimaeras, only a small population migrates into ventrolateral r1 (Fig. 4D), while the majority of migrating cells condense in the EGL. A few scattered cells lie within the pool of *Phox2a*-positive, presumptive locus coeruleus neurons. When *Hoxa2*-grafted r1 quail cells express Hoxa2, they condense in a dense cluster within ventrolateral r1 (Fig. 4E). The boundaries of their distribution are the same as the relatively small ventrolateral contribution in normal embryos. Despite the expanded rhombic lip contribution to ventral r1, the expression of *Phox2a* on control and operated sides of the brain is equivalent, suggesting that locus coeruleus formation is independent of changes in the population derived from the rhombic lip (Fig. 4E). Furthermore, while RCASBP(B)*Hoxa2* induces a profound change in the migration path, transfected rhombic lip derivatives are unlikely to account for the *Phox2a*-expressing neurons in the cerebellum following a global virus transfection.

Effects of Hoxa2 *on neuronal migration are rhombic-lip-autonomous*

The migratory behaviour of r1 rhombic lip cells can be altered by targeting *Hoxa2* overexpression to the precursor pool, suggesting that trajectory of its derivatives is at least partially determined by signals autonomous to the precursor pool. However, the environment through which cells migrate may also influence migration and condensation patterns. In particular, *Hoxa2* expression outside the rhombic lip may cause an accumulation of *Phox2a*-positive rhombic lip derivatives in dorsal r1 through a non-autonomous mechanism. To assess this possibility, normal quail cells were grafted orthotopically into dorsal r1 in chick embryos that had been electroporated with RCASBP(B)*Hoxa2* (*n* = 6). Figure 5A,5B,5C contrast, respectively, the contribution of rhombic lip cells to the EGL and ventrolateral r1 in three different combinations: control chimaeras, *Hoxa2*-transfected quail rhombic lip derivatives in a normal background, and normal quail derivatives in a *Hoxa2*-transfected background. While the EGL is depleted and a ventral component of the migration stream enhanced when r1 rhombic lip expresses *Hoxa2* (Fig. 5B), normal quail cells show equivalent migration on either a normal (Fig. 5A) or a *Hoxa2*-transfected background (Fig. 5C). This confirms that the regionally determined responses to migratory cues are determined autonomously within the rhombic lip.
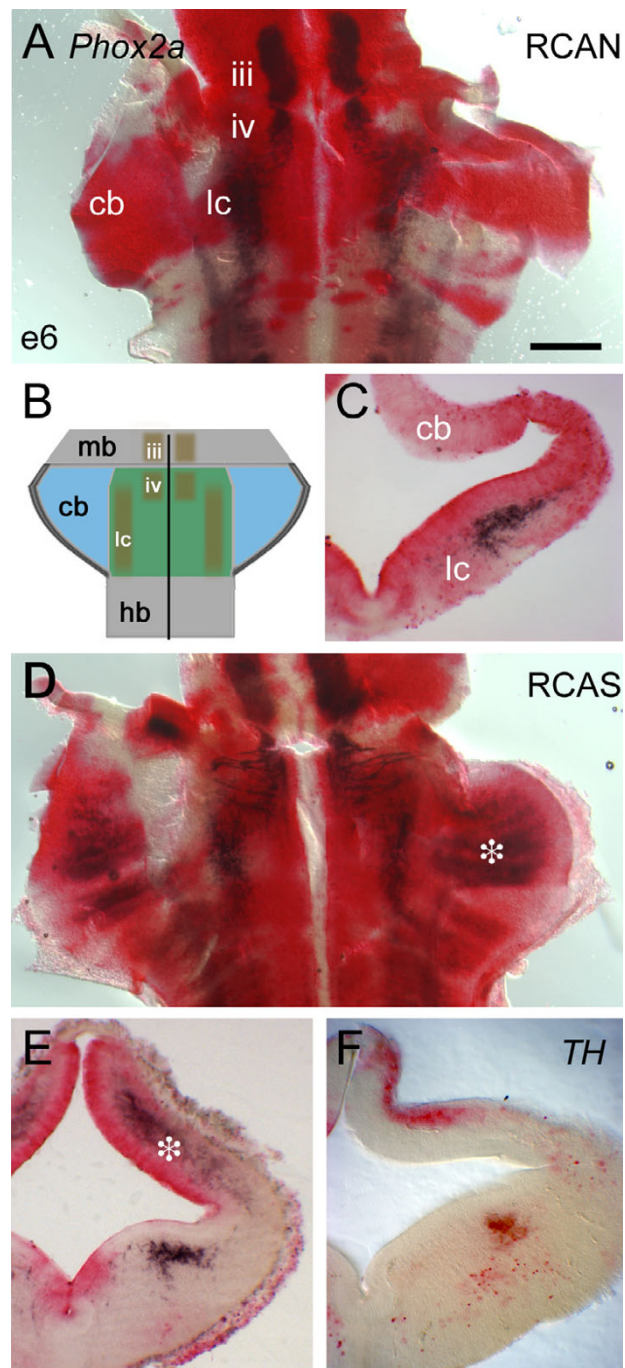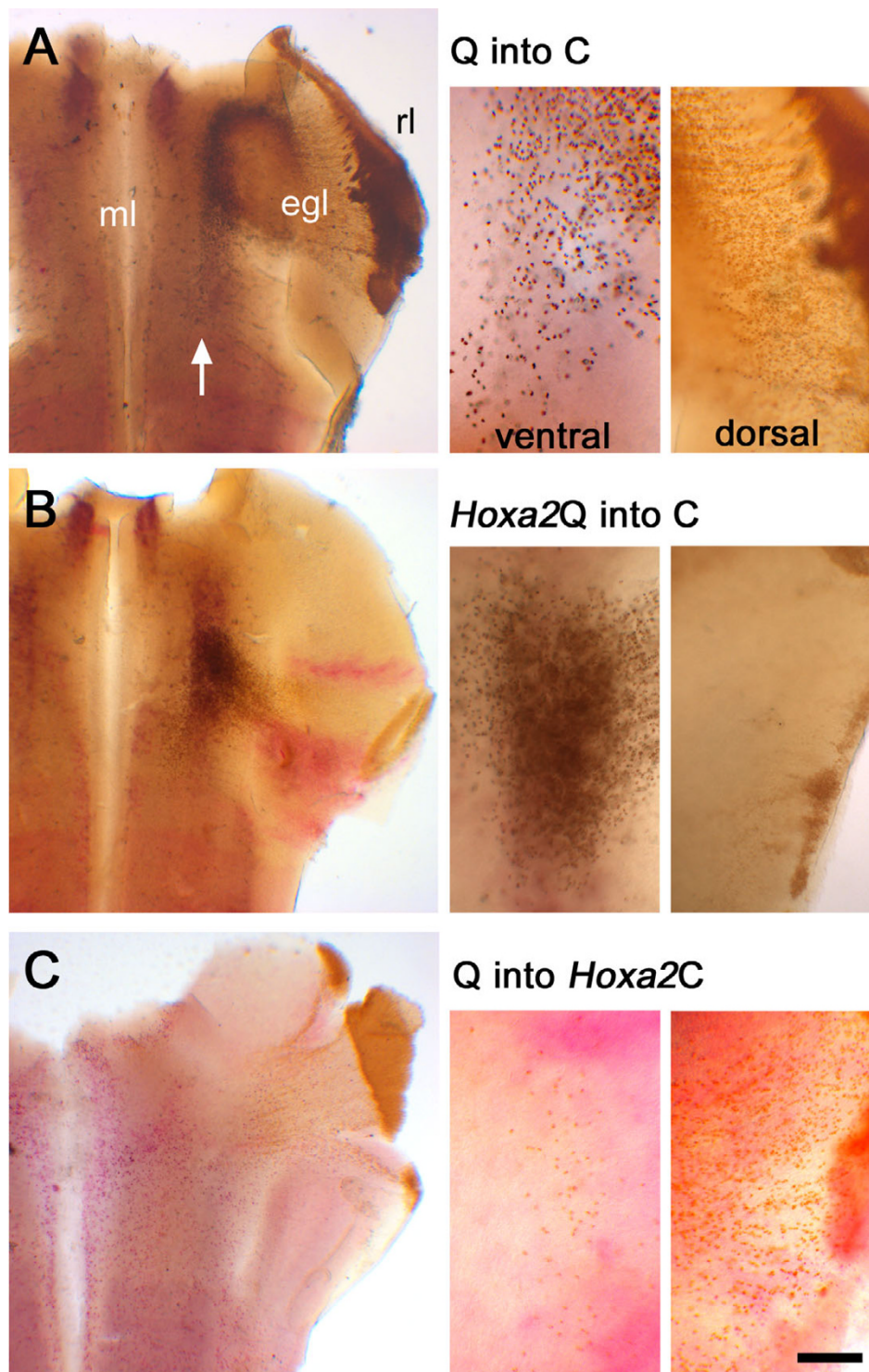
Figure 3
Locus coeruleus and trochlear nucleus formation is normal following global *Hoxa2* overexpression (A) Flat-mounted control embryo at embryonic day (E) 6, infected with RCANBP(B)*Hoxa2* virus (red); *Phox2a* expression (black) marks the oculomotor nucleus (iii) in the midbrain, and trochlear motor nucleus (iv) and locus coeruleus (lc) in rhombomere 1 (r1). There is no *Phox2a* expression within the cerebellar anlage (cb). **(B)** Schematic diagram showing the arrangement of *Phox2a*-positive nuclei within r1, as shown in (A). **(C)** This lack of dorsal expression is confirmed in transverse section, while the locus coeruleus is clearly identified by *Phox2a*. **(D)** *Hoxa2* up-regulation with RCASBP(B)*Hoxa2* (red) leads to ectopic *Phox2a* expression in the dorsal r1/cerebellum (asterisk). **(E)** In a transverse section slightly caudal to that in C, ectopic expression of *Phox2a* is confined to the deeper cerebellar layers (asterisk), while ventral expression of *Phox2a* in the locus coeruleus is unaltered. **(F)** *Hoxa2* up-regulation does not result in increased tyrosine hydroxylase (TH) staining within dorsal r1. Scale bar (A,D) = 400 μm. mb – midbrain, hb – hindbrain.
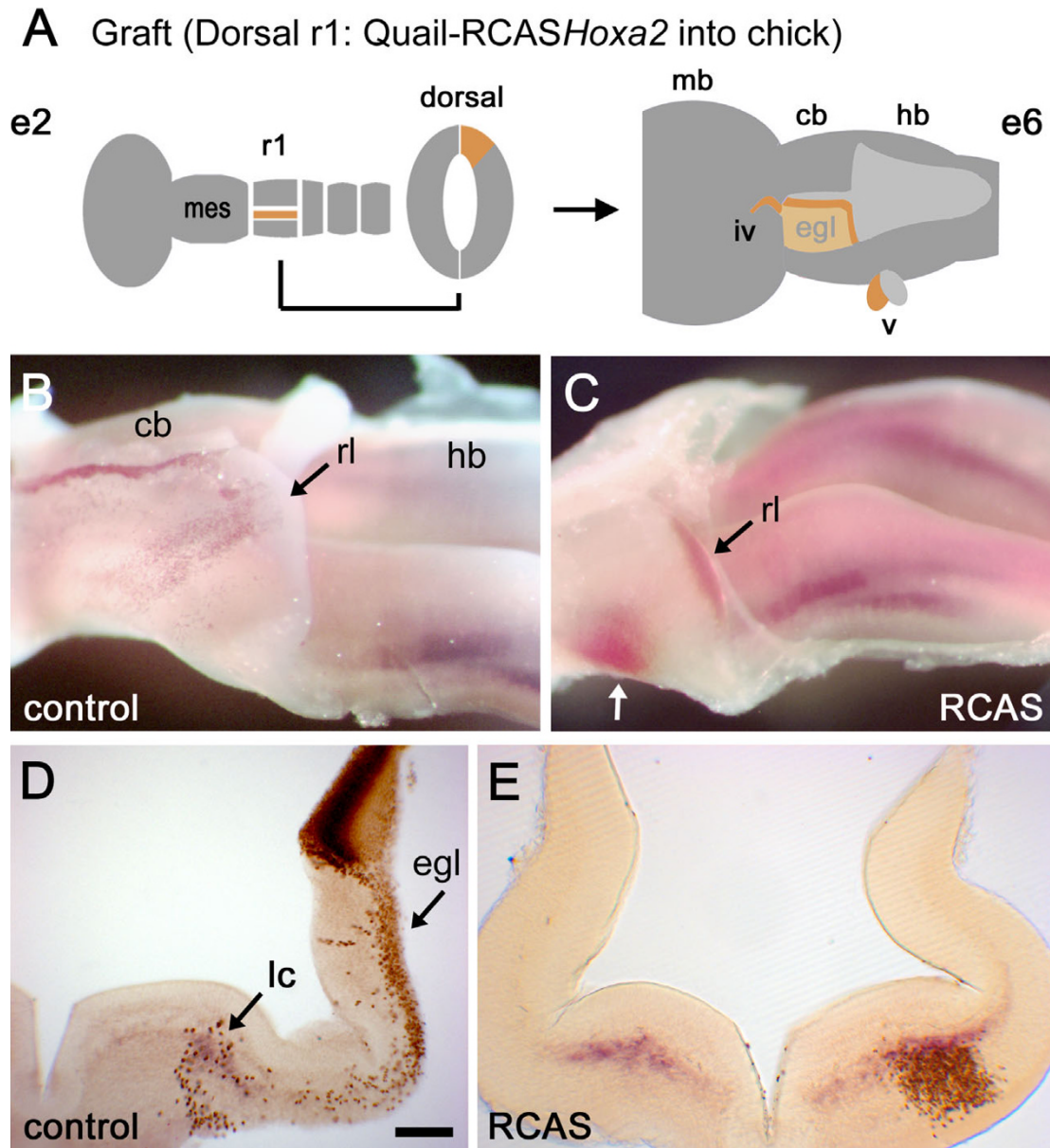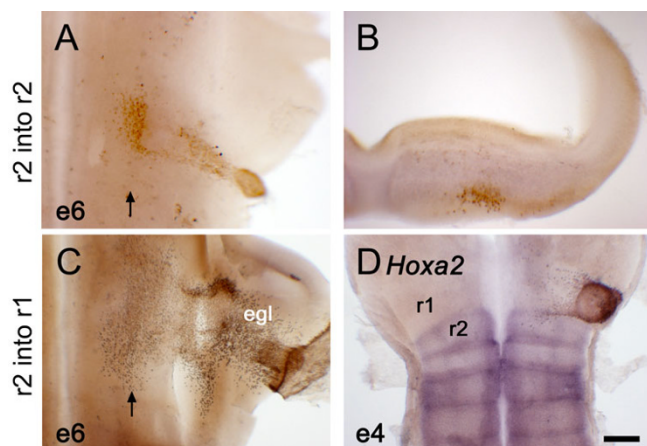
Figure 4

Targeted *Hoxa2* overexpression respecifies the migration of rhombic lip derivatives (A) Schematic diagram show-ing the location of donor tissue grafted from *Hoxa2* electroporated quail embryos at embryonic day (E) 2 (e2; left). A graft of dorsal rhombomere 1 (r1) maps to the cerebellar rhombic lip and roofplate at E6 (e6; right) and contributes to both the exter-nal granule cell layer (EGL) and neural crest derivatives in the trochlear (iv) and trigeminal (v) nerves [2]. **(B)** Lateral view of a wholemount dissection of the cerebellum (cb) and hindbrain (hb) of a control E6 chimaeric embryo stained with a quail-specific antibody, Q¢PN (brown), and stained for *Phox2a* (purple). The rostrocaudal axis runs left to right. A stream of ventrally migrat-ing quail cells originating from caudal rhombic lip (rl) lies over the presumptive cerebellum (point of origin indicated by a black arrow). A thin strip of quail cells also lies in the roofplate separating the bilateral anlage. **(C)** Migration patterns are substan-tially altered when grafted donor cells (black arrow) are electroporated with RCASBP(B)*Hoxa2*. Rhombic lip derivatives fail to condense in the EGL but rather migrate into a single ventrolateral nucleus (white arrow), which expresses high levels of ectopic *Hoxa2* (red). **(D)** Transverse section through the cerebellum of a control chimaera showing the donor progenitors in the dorsal neural tube. The majority of quail cell derivatives lie within the EGL (egl), with a scattering of cells ventrally overlap-ping the *Phoxa2* expression in the locus coeruleus (light blue). **(E)** By contrast, the majority of cells from grafts electroporated with *Hoxa2* condense ventrally (where quail cell nuclear diaminobenzidine label obscures the red *in situ* product marking *Hoxa2*). Scale bar (D,E) = 200 μm. mb – midbrain; mes – mesencephalon.

Figure 5
Effects on tangential migration of ectopic *Hoxa2* are rhombic-lip-autonomous A comparison of E6 quail-chick chimaeras with different combinations of targeted *Hoxa2* overexpression (red), in which the relative proportions of dorsal and ventral rhombic lip derivatives are compared (right). **(A)** Uninfected quail (Q) donor tissue in a normal chick (C) host (Q into C) gives rise to EGL cells and a small number of neurons in ventral r1 (white arrow). **(B)** Quail cells expressing *Hoxa2* in a normal host (Q-*Hoxa2*) bypass the EGL and condense ventrally. **(C)** Normal quail cells migrate in a host expressing ectopic *Hoxa2* (Q into *Hoxa2* C) migrate as controls. Scale bar for high-power insets = 100 μm. egl – external granule cell layer; ml – midline.

Figure 6
Derivatives of r2 dorsal neural tube have a characteristic migration pattern correlated to maintenance of endogenous *Hoxa2* expression (A) Flatmount of a fate-map chimaera at embryonic day (E) 6 (e6), in which quail r2 was orthotopically grafted into rhombomere 2 (r2) of a chick host. At E6, in flatmount, quail derivatives (brown) condense primarily in a single ventrolateral nucleus. **(B)** In transverse section, r2 migrants can be seen to settle in a subpial location similar to that of ventral derivatives in r1. **(C)** When transplanted into r1, quail r2 cells contribute to the EGL while maintaining a sparse ventral contribution. **(D)** When analysed by *in situ* hybridisation at E4 (e4), r2 grafted into r1 has lost its endogenous *Hoxa2* expression (blue). Scale bar = 100 μm.

Is the transformation of r1 rhombic lip by **Hoxa2** homeotic?

Previous studies have shown that the overexpression of vertebrate *Hox* genes in the hindbrain consistently results in transformations that can be described as homeotic, in that they recapitulate segmental characteristics of specific rhombomeres. *Hoxa2* chiefly regulates the identity of neurons in r2: thus, r1 rhombic lip cells transfected with *Hoxa2* might be expected to behave as r2 rhombic lip derivatives. In the absence of appropriate molecular markers for specific r2 populations, we used a grafting strategy to test this hypothesis.

We first assessed the normal migration path of r2 rhombic lip derivatives by grafting a narrow strip of dorsal quail r2 into dorsal chick r2 at E2, in ovo. In a significant fraction of the resultant chimaeras, analysed at E6 or E7, quail cells were found in the neural crest alone (*n* = 8/18), demonstrating that this grafting strategy effectively targeted the most dorsal cells of the neural tube [2]. In the remainder of the chimaeras, the grafted tissue was sufficiently large to generate migratory dorsal derivatives within the neural tube that condensed in a single ventrolateral nucleus, as

shown in flatmounted (Fig. 6A) and transversely sectioned (Fig. 6B) embryos. These derivatives were assumed to constitute the r2 rhombic lip derived population.

We then grafted equivalent dorsal quail r2 heterotopically into r1, to directly compare the behaviour of r2 rhombic lip derivatives with that of r1 derivatives infected with *Hoxa2* (Figs. 4, 5). In contrast to orthotopically grafted tissue, the distribution of r2 quail cells within r1, analysed at E6 (*n* = 6), more closely resembled that of normal r1 rhombic lip derivatives. Rhombic lip from r2 contributed to both the EGL and a ventrolateral nucleus (Fig. 6C). The difference in behaviour between r2 dorsal derivative within r2 (Fig. 6A) and r1 (Fig. 6B) can be attributed to the respecification of r2 precursors grafted into r1. *In situ* analysis of heterotopically grafted r2 demonstrates that, as early as E4 (*n* = 7), endogenous *Hoxa2* expression is down-regulated in donor quail cells (Fig. 6D), presumably by signals from the isthmus [24,25]. Thus, it is impossible within r1 to directly compare the migration path of rhombic lip derivatives from *Hoxa2*-infected r1 with those from r2 (Fig. 6A,6B). However, *Hoxa2* overexpression within the cerebellar rhombic lip results in a rhombic lip migration path reminiscent of that in r2 (Fig. 6A,6B), supporting a conclusion that the transformation is homeotic. Furthermore, the maintenance of an r2-specific migration pattern is correlated with the maintenance of endogenous *Hoxa2* expression.

Discussion
We have used a combination of viral overexpression and electroporation coupled with grafting to assess the effects of ectopic *Hoxa2* on selected features of r1. Viral infection allowed for the study of early-onset gene transfection on patterning of anterior hindbrain. Nonspecific effects of RCAS infection, reported for electroporated embryos [26], were controlled for by injections RCAN virus, which lacks a splice acceptor and thus produces mRNA but no protein. At later stages, a combination of electroporation into quail embryos and microsurgery allowed us to target overexpression to specific precursor pools. The results of these different technical approaches revealed a clear division within r1 development between precursors that are sensitive and those that are refractory to *Hoxa2* overexpression (Fig. 7). These observations were consistent for different degrees of transfection, suggesting that our results are unlikely to reflect a differential response by r1 precursors to varying *Hox* gene dosage. Rather, we interpret this observation as reflecting divergent patterning strategies within r1 for different neuronal groups, as revealed by an all-or-nothing sensitivity to ectopic *Hoxa2*.

Features of r1 that are unaffected by ectopic **Hoxa2**
We found that the initial allocation of r1 territory, the expression of isthmic signalling genes *FGF8*, *Wnt1* and
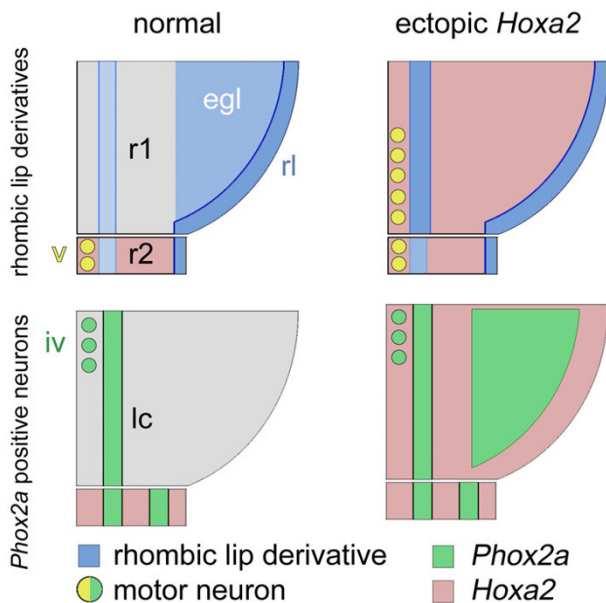
Figure 7
Summary of the effects of *Hoxa2* overexpression on r1 derivatives The arrangement of rhombic lip derivatives in rhombomere 1 (r1) (top) and *Phox2a*-positive neurons (bottom) shown on a schematised flatmount of a half neural tube from normal (left) and *Hoxa2*-infected (right) embryos. **(Top)** *Hoxa2* overexpression in r1 results in the loss of the EGL and an expansion of ventral rhombic lip derivatives. Ectopic trigeminal-like motor neurons are generated in caudal r1 [14]. **(Bottom)** The trochlear nucleus and locus coeruleus form normally when *Hoxa2* is overexpressed; however, dorsal r1 expresses high levels of ectopic *Phox2a*. The cells of this latter population are neither TH-positive locus coeruleus precursors that have stalled during migration nor rhombic lip derivatives that have altered their fate. egl – external granule cell layer; rl – rhombic lip.

en1, the formation of the trochlear nucleus and the establishment of the locus coeruleus were unaffected by ectopic *Hoxa2* expression within r1. By using retrovirus injection into pregastrula embryos, overexpression was induced before the establishment of either an *Otx2*-, *Hoxa2*-negative domain (at st.6) or isthmic organiser (st.8; [27]). Thus, ectopic *Hoxa2* can be presumed to have no influence on the inductive interactions between midbrain (*Otx2*-positive) and hindbrain (*Gbx2*-positive) territory that establish isthmic signalling [3,4]. While FGF8 can repress *Hoxa2* in the hindbrain (Fig. 6B; [24,25]), the converse is not found at the isthmus.

Isthmic signalling plays a role in regulating the development of r1 [3,6,8,24] where FGF8 is sufficient for the induction the trochlear nucleus [8] and necessary for

locus coeruleus specification [9]. These processes, which are overtly linked to isthmic activity, are refractory to experimental *Hoxa2* up-regulation, suggesting that a complex of isthmic induction pathways in r1 is independent of a set of patterning processes that rely on segmental identity cues.

Structures affected by *Hoxa2* overexpression
By contrast, various aspects of r1 specification and patterning are profoundly influenced by ectopic *Hoxa2*. In a previous study, RCASBP(B)*Hoxa2* was shown to induce an r2-specific motor neuron phenotype within ventral r1 [14], caudal to the trochlear nucleus, in a region normally devoid of any motor efferents. Ventral progenitors were thus presumed to undergo a homeotic transformation to a more caudal fate (Fig. 7).

Within dorsal r1, ectopic *Hoxa2* results in a smaller cerebellum, consistent with the loss of the EGL due to the aberrant migration of granule cells to a ventrolateral target. Both the loss of specific granule cell markers and the induction of a pattern of nuclear condensation corresponding to that of r2 derivatives suggest that rhombic lip precursors have been caudalised in a homeotic manner. Furthermore, a combination of grafting and electroporation reveals that this effect is independent of changes to r1 as a whole. This suggests that segmental/ rhombomeric identity programmes rhombic lip derivatives with region specific responses to generalised dorsoventral migration cues such as netrin [28,29] and slit [23].

This transformation of rhombic lip migration pattern is independent of the formation of a cell-free layer corresponding to the EGL (Fig. 2F,2G), in distinct contrast to mouse models of granule cell depletion, such as the MATH1 mutant, where the EGL fails to form [30]. This indicates that the transient presence of *Hoxa2*-positive rhombic lip derivatives migrating over the cerebellar anlage is apparently sufficient to trigger the allocation of space for an EGL, even if this layer is subsequently unoccupied.

Our observations cast doubt on lineage analyses implicating the rhombic lip as the origin of locus coeruleus neurons [22]. Despite dramatic alterations in migration and condensation patterns of rhombic lip derivatives, the locus coeruleus always forms correctly. Furthermore, *Hoxa2* induced, ecoptic *Phox2a*-positive neurons, which seemed ideal candidates for the 'lost' EGL population, have an as yet indeterminate origin. Our results correspond with observations indicating that locus coeruleus neurons are born earlier than EGL precursors [21] from a *Mash1*-positive precursor pool [31], which lies ventral to the *Math1*-positive rhombic lip [32].

Finally, the fate of other neuronal types born within dorsal r1, including the remainder of the cerebellum [33], remains to be determined. Ectopic dorsal *Phox2a* is seen in globally infected embryos but not when *Hoxa2* is targeted to the rhombic lip (Fig. 4E). This points to further transformations of cerebellar neurons when *Hoxa2* is overexpressed throughout dorsal r1 that are not secondary to the loss of granule cells. However separating autonomous and non-autonomous effects of ectopic *Hoxa2* in dorsal r1 will require an accurate fate-map of the origins of each cerebellar neuronal population. With the exception of granule cells, the dorsoventral origins of each cerebellar neuronal type have not yet been established.

Origins of the cerebellum: segmental identity versus organiser activity

While midbrain/hindbrain patterning has focused on the role of the isthmic organiser in regional patterning [5,24,25], *Hoxa2* overexpression reveals a dependence on segmental identity for the specification of cerebellar granule cells. It thus seems likely that the establishment of a specific *Otx2*-, *Hoxa2*-negative region at st.6 (Fig. 1A) is an important step in the specification of the cerebellum. Consistent with this hypothesis, the loss of *Hoxa2* expression in r2 leads to a caudal expansion of the cerebellum [10], presumably in the absence of any changes in isthmic signalling cues. These results suggest that cerebellar granule cell induction is independent of isthmic signalling per se. Our results agree with suggestions that the effects of experimental manipulation of FGF on cerebellar induction act through modulation of *Otx2* [5,7], rather than a direct induction. Thus, the induction of ectopic cerebellar neurons by exogenous FGF application can be attributed to the down-regulation *Otx2* in the midbrain [6,7] or *Hoxa2* in the hindbrain [24], recapitulating the molecular characteristics of r1. Correspondingly, loss of FGF signalling in the zebrafish *ace* mutant leads to an expansion of *Otx2* within r1 territory, suppressing granule cell induction [5].

The establishment of r1 is therefore necessary for cerebellar induction and, by molecular criteria, precedes that of any detectable isthmic organiser. Whether the absence of both *Otx* and *Hox* genes is sufficient to potentiate cerebellar development in the absence of isthmic signalling is unclear. Complete suppression of *Hox* gene function results in a hindbrain that adopts a 'default' r1 phenotype in zebrafish [13], but whether later dorsal development is 'cerebellar' has not been determined. As in ontogeny, the appearance of r1 precedes, phylogenetically, that of both the isthmic organiser [34] and the cerebellum [35]. The evolution of a cerebellum would therefore seem to require a modification of either extrinsic signalling cues or responses within r1, in addition to the simple allocation of r1 territory.

Conclusions

Our results suggest that rhombomere 1 displays patterning influences that are characteristic of both the isthmic organiser and the rhombomeric organisation of the hindbrain. Using *Hoxa2* overexpression to repattern regional identity of r1, rhombic lip derivatives that give rise to granule cell precursors can be forced to undergo a radical reprogramming of their migration and fate. By contrast, isthmic signalling molecules and both the territorial extent and the establishment of a subset of r1 nuclei are refractory to segmental cues and thus are presumed to be patterned independently by the midbrain/hindbrain organiser. This supports an argument that granule cells are a product of the molecular identity of r1 and induced in an isthmus-independent manner.

Methods
Viral overexpression of Hoxa2

Chick eggs (Hi-Sex White) were windowed with scissors at either E0 (6 hours of incubation) or E2 (stage [st.] 8$^+$ to 10$^-$, [36]). The anterior neural tube was injected with the retrovirus encoding *Hoxa2*, RCASBP(B)*Hoxa2* (10^8 to 10^9 virions/ml) To control for the nonspecific effects of viral infection, a different group of embryos were injected with the retrovirus, RCANBP(B)*Hoxa2*, which lacks a splice acceptor site immediately 5' of *Hoxa2* and hence fails to produce functional Hoxa2 protein [14]. Eggs were sealed with tape and reincubated at 37°C for 4 to 7 days.

Electroporation and microsurgery

Donor quail embryos were windowed at E2 and plasmid containing the proviral sequences of RCASBP(B)*Hoxa2* (1 mg/ml in water containing 0.0015% fast green) was injected into the neural tube at the level of r1/2. Cells were then electroporated (1–2 pulses, 200 milliseconds/10 V, square waveform) using electrodes placed ventral and dorsal to the donor r1. These and a control group of unelectroporated donor embryos were harvested and the anterior neural tube isolated in Tyrode's solution (137 mM NaCl, 2.7 mM KCl, 1.8 mM $CaCl_2$, 3.5 mM NaH_2PO_4, 0.1 mM $MgCl_2$, 5.5 mM D-glucose). The dorsal part of r1 was dissected away, after treatment with dispase (Roche, 1 mg/ml in L15, 10 minutes), using flame-sharpened tungsten wire [37]. Tissue from both donor groups was orthotopically grafted into stage-matched host chick embryos, which were then sealed and reincubated for 4 or 5 days. In a complementary strategy, unelectroporated quail dorsal r1 was grafted into chick hosts that had been electroporated with RCASBP(B)*Hoxa2*. In a separate set of fate-mapping experiments, dorsal r2 from quail was grafted orthotopically into r2 or heterotopically into r1 of host chick embryos, which were reincubated for 2 to 5 days.

In situ *hybridisation and immunohistochemistry*

Virally infected, chimaeric embryos and controls were harvested at E6 in 4% paraformaldehyde in 0.01 M phosphate buffer (PFA) at 4°C. The cerebellum and hindbrain region were partially dissected and processed for *in situ* hybridisation as described elsewhere [38]. Plasmid templates from a number of sources were used to generate digoxygenin- (dig-) and FITC-labelled antisense riboprobes: *Hoxa2* [39], *Phox2a* (from P Goridis, INSERM, Marseilles), *erbB4* [19], *Pax6* [40], TH [21], *en1* [41], *Wnt1* (Dr Anthony Graham, King's College London, UK), *FGF8* [27], *Gbx2* [42] and *Otx2* [17].

Quail–chick chimaeras were further stained using an antibody, Q¢PN (DSHB, Iowa University, Iowa, USA) to a quail-specific perinuclear marker and a peroxidase-conjugated secondary antibody. Embryonic brains were sectioned transversely at 50 μm on a vibratome, mounted on slides in glycerol/PBS (9:1) and photographed using an Axiocam mounted on a Zeiss Axiophot.

Authors' contributions

ME and LT contributed equally to studies of the overexpression of *Hoxa2* and its analysis. EB characterised early gene expression in the midbrain and hindbrain. RJTW planned the study, performed the microsurgery and analysed the results. All the authors have read and approved the manuscript.

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