# Rostral optic tectum acquires caudal characteristics following ectopic *Engrailed* expression

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**Background**: Expression of the homeobox-containing gene *Engrailed* (*En*) in an increasing rostral-to-caudal gradient in the dorsal mesencephalon is the earliest known marker for polarity of the chick optic tectum. In heterotopic transplantation experiments, En protein expression correlates well with the subsequent gradient of cytoarchitecture as well as the pattern of retinotectal projections. The En gradient also correlates with the expression of two putative retinal axon-guidance molecules, RAGS and ELF-1, which are Eph-like receptor tyrosine kinase ligands that may function in the establishment of retinotopic projections by excluding temporal axons from the caudal tectum.

**Results**: To examine the function of *En* in determining tectal polarity, we used the replication-competent retroviral vector RCAS to misexpress mouse *En-1* throughout the chick tectal primordium. Our results show that the rostral portion of the tectum adopts a caudal phenotype: the gradient of cytoarchitectonic differentiation is abolished, and the molecular markers RAGS and ELF-1 are strongly expressed rostrally. In addition, cell membranes from rostral tectum of RCAS *En-1*-infected embryos preferentially repel temporal axons in *in vitro* membrane stripe assays.

**Conclusions**: These results are consistent with a role for *En* in determining rostrocaudal polarity of the developing tectum. The demonstration that both RAGS and ELF-1 are upregulated following En misexpression provides a molecular basis for understanding the previous observation, also based on retrovirus-mediated En misexpression, that nasal axons form ectopic connections in rostral tectum, from which temporal axons are excluded.

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## Background

The question of how positional information is encoded in the vertebrate nervous system is of fundamental importance in developmental neurobiology. During neural plate induction, a number of putative developmental control genes become expressed in spatially restricted patterns along the anteroposterior axis of the developing central nervous system (CNS), suggesting that they play a part in specifying the fate of distinct CNS regions. Amongst these are two homeobox-containing genes, *En-1* and *En-2* (reviewed in [1]), which are homologues of the *Drosophila* segmentation gene *engrailed* (*en*) [2,3]. In mouse, *En-1* and *En-2* are expressed from the one- and five-somite stages, respectively [4–6], before any overt signs of region-specific morphogenesis. Their mutually overlapping expression domains in the rostral neuroepithelium span the future caudal mesencephalon and rostral rhombencephalon, and involve cells whose ultimate fate includes formation of the caudal half of the optic tectum. The function of *En* in vertebrate CNS development has been investigated by gene-targeting experiments, which show that En is crucially involved in the early morphogenetic specification of the CNS. Homozygous *En-1* mutant mice [7] have severe deletions of the midbrain and cerebellum, encompassing the entire domain of *En* expression. Homozygous *En-2* mutant mice [8,9], however, display much less severe abnormalities, which are limited to foliation of the cerebellum. Recent analysis has shown that the contrasting phenotypes of these null mutant animals reflect differences in the temporal and spatial expression of the two mouse En proteins and not a divergence in their biochemical activities. Thus, the *En-1* mutant phenotype can be completely rescued following insertion of mouse *En-2* coding sequences into the *En-1* locus [10]. Furthermore, partial rescue was also obtained using *Drosophila en*, suggesting that the functional activity of En proteins has been at least partially conserved through evolution. The overlapping expression and function of the two mouse *En* genes, together with the loss of dorsal midbrain tissue in both the *En-1* knockout and the double *En-1*/*En-2* knockout mice [11], means that existing mouse mutants cannot be used to examine the later role of En in the development of midbrain polarity.

In chick embryos, immunohistochemical analyses of En expression have been carried out using a polyclonal antiserum, αEnhb-1, which detects both En-1 and En-2 [5], and a monoclonal antibody, 4D9, which specifically detects En-2 [12–15]. En expression begins in the presumptive mesencephalic/rhombencephalic region at the four-somite stage, and later forms a gradient in the neuroepithelium that decreases both rostrally and caudally from the midbrain–hindbrain constriction. As in mouse, En-2 expression in chick lags slightly behind that of En-1 and persists in a number of mesencephalic and rhombencephalic derivatives, including the optic tectum, cerebellum and several nuclei within the mes-isthmo-cerebellar region [15]. In the dorsal mesencephalon, En-2 expression is restricted to the postmitotic cells in the caudal region of the optic tectum. At embryonic day 10 (E10), the level of expression and density of *En-2*-labelled cells differs between the tectal layers: two deep layers — the stratum griseum periventriculare and stratum griseum centrale — express high levels of protein, whereas the superficial sublayers — the stratum griseum et fibrosum superficiale (SGFS) — contain the highest number of labelled cells. At later stages, expression persists in only the most superficial layers of the SGFS.

The gradient of En expression in the mesencephalon at E2 is the earliest known marker for tectal polarity. Later in development, the gradient is matched by a pronounced difference in cytoarchitecture, whereby the rostral tectum has a thicker wall and more laminae than the caudal tectum [16]. Later still, the rostral and caudal regions of the tectum acquire different sets of afferent inputs from the retina: axons extending from ganglion cells in the temporal retina innervate the rostral tectum, whereas the caudal tectum receives axons from the nasal retina [17,18]. Studies aimed at discovering the molecular basis of retinotopic map formation have recently identified putative axon-guidance molecules that are expressed in decreasing caudal-to-rostral gradients in the chick tectum, reminiscent of the earlier pattern of En expression. Two molecules in particular, RAGS [19] and ELF-1 [20], both of which are ligands for EPH-like receptor tyrosine kinases, are expressed strongly in the caudal tectum where they may function to inhibit temporal axon ingrowth and/or to attract nasal axons.

Transplantation studies using chick–quail chimeras have shown that the induction and/or maintenance of En expression in neuroepithelial grafts correlates well with later morphological development into midbrain–hindbrain structures (reviewed in [21]). Further studies have shown that En expression in an increasing rostrocaudal gradient in the caudal half of the dorsal mesencephalon correlates well with the subsequent rostrocaudal polarity observed in the optic tectum (reviewed in [22]). Thus, when the mesencephalic vesicle is reversed on the anteroposterior axis at E2, the En gradient re-adjusts to its original polarity [23] and both the graded cytoarchitecture and pattern of retinotectal projections develop normally [24–26]. When the reversal is done at E3, however, the En gradient does not adjust [26], and cytoarchitecture and retinotectal projections are subsequently inverted [25,26]. The correlation between early En expression and the pattern of retinal axon ingrowth has been recently further strengthened by experiments in which *En* was misexpressed in the rostral tectum using a retroviral vector. These results showed that, in infected embryos, the guidance of retinal axons was perturbed: in some cases, nasal axons arborized ectopically in the rostral tectum, whereas temporal retinal axons failed even to enter the tectum [27].

To investigate the role that *En* plays in determining the rostrocaudal polarity of the developing tectum, we have used the replication-competent retroviral vector RCAS [28] to misexpress mouse *En-1* within the chick tectal primordia. Here, we show that the rostral portion of the tectum adopts a caudal phenotype following infection of the anterior neuroepithelium at early embryonic stages, as assessed by morphological and molecular changes, as well as changes in the behaviour of retinal axons.

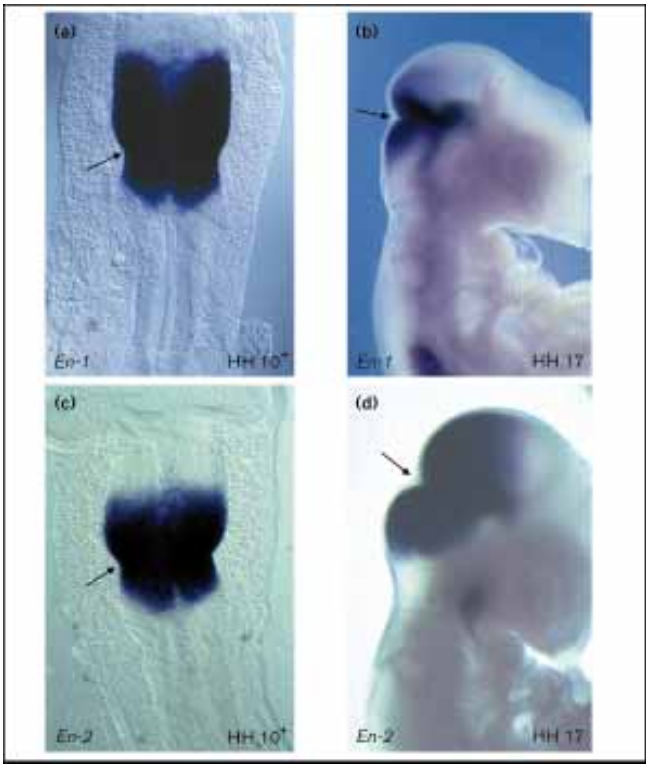
## Results and discussion

### Misexpression of *En-1* in the developing tectum

Using gene-specific probes in whole-mount RNA *in situ* hybridization analyses, we found that the two chick *En* genes, *En-1* and *En-2* [29], were expressed from the three- and six-somite stages, respectively, in overlapping domains in the presumptive mesencephalic/rhombencephalic region (C.L., H. Sheikh, I. Mason and A.L., unpublished observations); this spatiotemporal pattern is comparable to that previously seen in the mouse [4–6]. As shown in Figure 1, *En-1* transcripts initially extend further rostral than those of *En-2*. *En-1* transcripts, however, unlike *En-2* transcripts, were not detected in the developing tectum at E9 (data not shown).

To modify the En expression pattern in the developing tectum, a mouse *En-1* cDNA [29,30] was inserted into the replication-competent retroviral vector RCASBP(A) [28], generating RCAS *En-1*. The two mouse *En* genes have been shown previously to be functionally equivalent to those of other species, including chick [31] and *Drosophila* [10], and to be functionally equivalent to each other [10]. Concentrated viral supernatant containing RCAS *En-1* was injected into the developing neural tube or layered on top of the cephalic neuroepithelium of embryos at Hamburger and Hamilton (HH) stages 7–9 *in ovo*. Ectopic *En-1* expression was subsequently detected by whole-mount RNA *in situ* hybridization, and distinguished from endogenous *En-1* expression using a mouse-specific probe. En protein was detected by immunohistochemistry using the αEnhb-1 antiserum [5], which recognizes both En proteins in mouse and in chick.

**Figure 1**




Whole-mount RNA *in situ* hybridization analysis of endogenous *En-1* (a,b) and *En-2* (c,d) expression. (a,c) Dorsal view of 11-somite (Hamburger and Hamilton (HH) stage 10$^+$)embryos. (b,d) Lateral view of HH stage 17 embryos. Although *En-1* expression (blue) as shown in (a) initially extends further rostral than *En-2* (c), it later becomes more restricted (b,d). *En-2* expression, unlike that of *En-1*, persists within the developing tectum at E10 (data not shown). The constriction between midbrain and hindbrain is marked by the arrows.

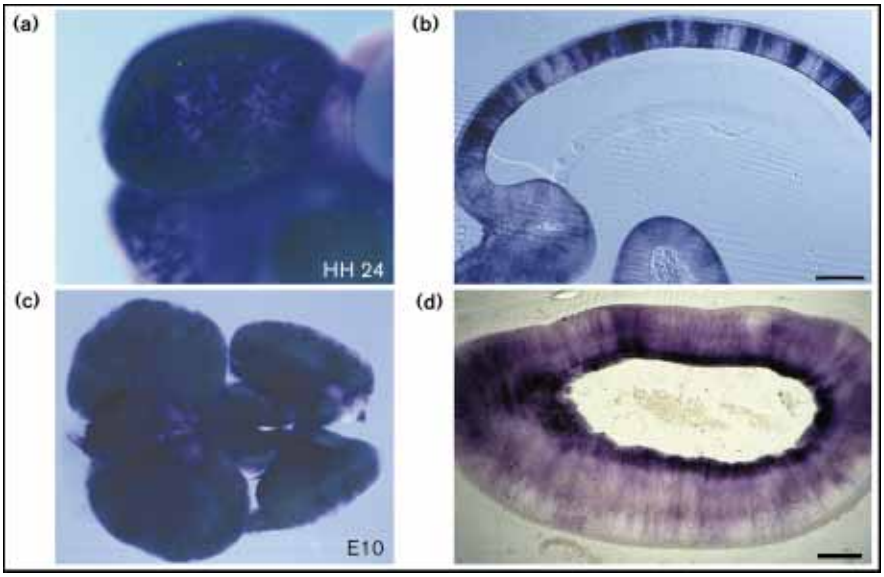
By HH stage 24, approximately 30–60 % of the cells in the mesencephalon expressed mouse *En-1* (Fig. 2a,b). At E10, patches of exogenous *En* expression typically covered more than 50 % of the tectum and were equally distributed along the rostrocaudal axis (Fig. 2c,d). Similar expression patterns were seen using a control vector, RCAN *En-1*, which lacks the splice acceptor required for proper translation of En protein (data not shown). The overall brain morphology of embryos heavily infected with either construct appeared normal.

### The rostrocaudal gradient of cytoarchitectonic development is abolished following *En-1* misexpression

From about E5 onwards, there is a pronounced rostrocaudal gradient of cytoarchitecture in the normal chick optic tectum [16] — the rostral region of the tectum differentiates faster, has a greater overall thickness and manifests a more advanced laminar structure than the caudal region. To determine whether the pattern of En expression directly correlates with the subsequent rostrocaudal gradient of cytoarchitectonic differentiation, as has been suggested from transplantation experiments using chick–quail chimeras [22,25,26,32], we examined the cytoarchitecture in E10 tecta ectopically expressing En following viral infection at E1.5. If En is involved in this process, we would expect En-expressing regions to be thinner overall and to have a more immature laminar structure than regions not expressing En.

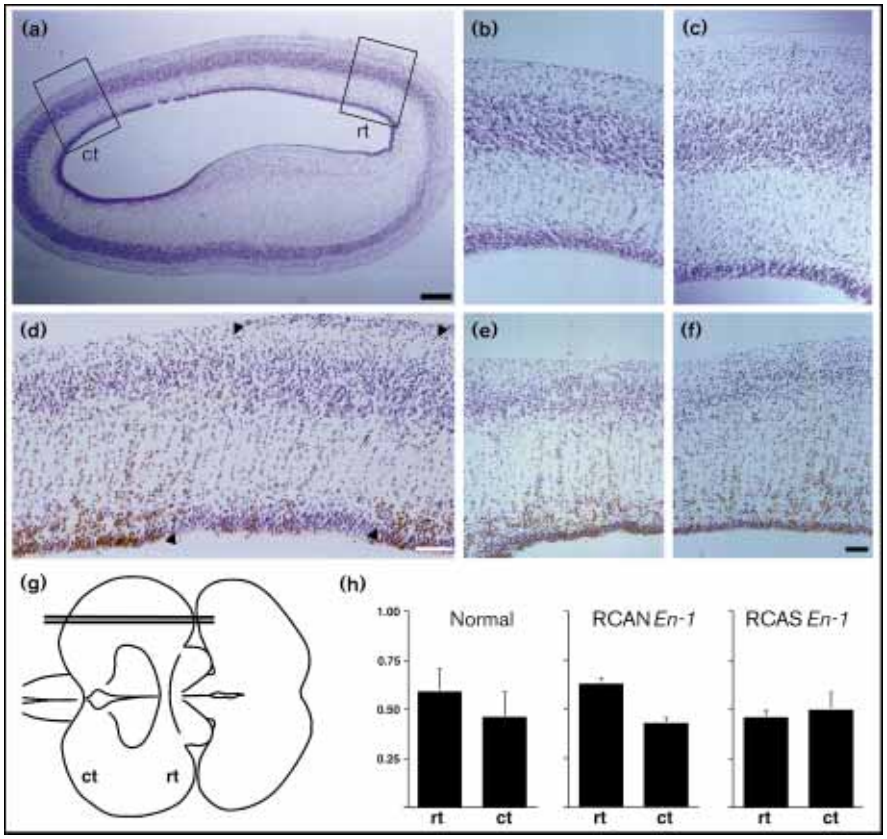
At E10, rostrocaudal differences in both thickness and lamination were clearly discernible in normal (uninfected) embryos (Fig. 3a–c). At this stage, the caudal tectum, which had previously expressed high levels of En protein, was thinner and contained fewer discernable layers than

**Figure 2**




Whole-mount RNA *in situ* hybridization analysis of ectopic mouse *En-1* expression (blue) following retroviral infection at HH stages 8–9. (a) Lateral view of a HH stage 24 embryo. (c) Dorsal view of partially dissected E10 brain. (b) and (d) are parasagittal sections through (a) and (c), respectively. Rostral is to the right in all panels. Scale bars: (b) = 60 μm; (d) = 300 μm.

**Figure 3**



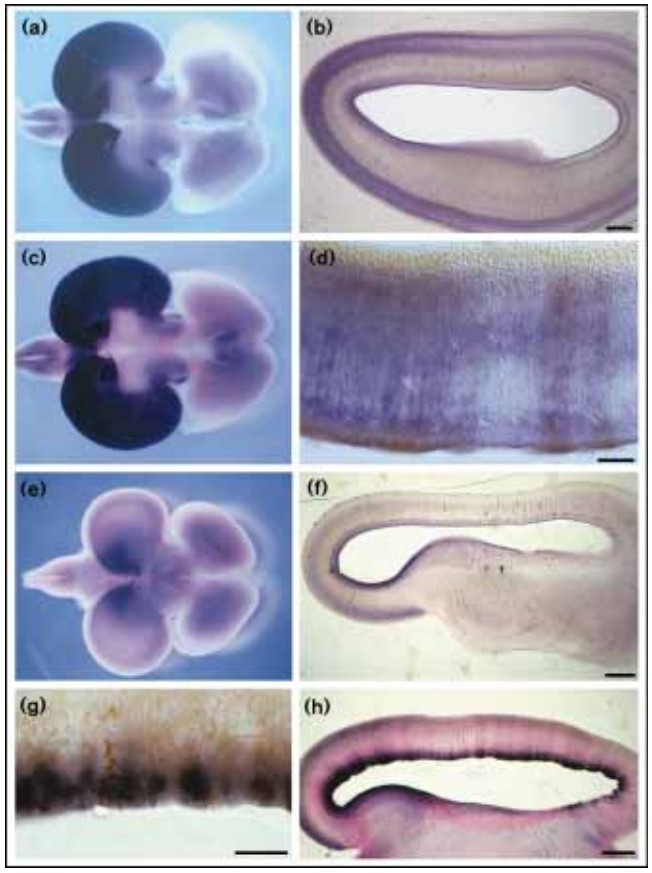

Cytoarchitecture of normal (a–c) and RCAS *En-1*-infected E10 tecta (d–f) following retroviral infection at HH stage 8. Rostral is to the right and caudal to the left in all panels. All sections are from the parasagital plane shown in (g) and have been immunostained using αEnhb-1 to detect En protein (brown) and counterstained with cresyl violet. Ectoptic En protein is superimposed on the normal medial-to-lateral decreasing gradient of En-2 expression within the caudal tectum. Endogenous En-2 expression, however, is not detectable within the plane of section shown. High-magnification views of caudal (b,e) and rostral (c,f) tectum are from the regions boxed in (a). (d) Another region through RCAS *En-1*-infected rostral tectum, showing spatial correlation between ectopic En expression (brown) and tectal cytoarchitecture. Arrowheads delineate transition between regions expressing high and low levels of En. (h) Schematic representation of the thickness (in mm) of the rostral tectum (rt) and caudal tectum (ct) in normal (uninfected; $n = 11$), RCAN *En-1*-infected ($n = 3$) and RCAS *En-1*-infected ($n = 10$) tectum at E10. All measurements were taken from approximately the same dorsolateral region, as indicated in (a) and (g). Scale bars: (a) = 300 μm; (f) = 60 μm. (b,c,e) are at the same magnification as (d,f).

the rostral tectum. A similar gradient was seen in embryos infected with the control vector, RCAN *En-1*. As shown schematically in Figure 3h, the rostral region in both uninfected (normal) and RCAN *En-1*-infected (control) tecta was on average 30 % thicker than the corresponding caudal region. In contrast, the normal gradient of cytoarchitectonic differentiation was not detectable and/or irregular in tecta ectopically expressing En (Fig. 3d–f). We found no differences in the overall thickness between the rostral and caudal tectum in RCAS *En-1*-infected tecta uniformly expressing En at E10 (Fig. 3h). Close examination, however, revealed that rostral patches strongly expressing En were thinner and had fewer discernable layers than adjacent patches of rostral tectum where En was not expressed (Fig. 3d). Overall, the rostral region of RCAS *En-1*-infected tecta was thinner than the rostral region of normal (uninfected) and RCAN *En-1*-infected controls, had fewer discernible layers and was therefore presumably in a less advanced state of differentiation. Indeed, the thickness and less advanced laminar structure observed in RCAS *En-1*-infected tecta more closely resembled that seen in the caudal region of uninfected and RCAN *En-1*-infected controls. These results provide direct evidence that En expression can influence the rate of cytoarchitectonic development, with possible influences on proliferation and/or migration. Furthermore, they suggest that ectopic expression of *En-1* in the rostral tectum results in an anterior-to-posterior transformation.

### ELF-1 and RAGS are ectopically expressed

To determine whether the rostral tectum had acquired characteristics of the caudal tectum, we examined the expression of ELF-1 and RAGS, which are normally expressed in a decreasing caudal-to-rostral gradient in the tectum (Fig. 4a,b,e,f) in patterns similar to, but much later in development than, that of En. Following infection with RCAS *En-1*, both genes were strongly expressed in the rostral as well as caudal tectum in E10 embryos (Fig. 4c,d,g,h), suggesting that the rostral tectum had indeed adopted a caudal phenotype. Interestingly, we did not detect ectopic expression of ELF-1 and RAGS in other regions of the brain that expressed exogenous *En-1*, and neither gene was expressed *in vitro* in chick embryo fibroblasts transfected with, and expressing, RCAS *En-1* (data not shown). Hence, the competence of cells to express ELF-1 and RAGS in response to En expression seems to be limited to the tectal field itself. Furthermore, within the tectum, ELF-1 and RAGS seemed to be ectopically expressed mainly within appropriate laminae — namely, RAGS was strongly expressed in the innermost

**Figure 4**




Whole-mount RNA *in situ* hybridization analysis of ectopic ELF-1 and RAGS expression at E10 following retroviral infection at HH stages 8–9. Rostral is to the right in all panels. Endogenous expression patterns (blue) of ELF-1 and RAGS are shown in (a,b) and (e,f), respectively. (a,e) Dorsal view of partially dissected E10 brain. (b,f) Parasagital section through (a) and (e), respectively. Both ELF-1 and RAGS are normally expressed in a decreasing caudal-to-rostral gradient within the tectum. Pattern of ELF-1 (c,d) and RAGS (g,h) expression (blue) in the tectum of RCAS *En-1*-infected embryos. The pattern of ectopic ELF-1 and RAGS expression is superimposed on the normal caudal-to-rostral gradient of expression. (c) Dorsal view of partially dissected E10 brain. (d) Parasagital section through the rostral portion of (c) counter-immunostained for ectopic En protein (brown). At E10, endogenous En protein is not detected within the rostral tectum (data not shown). (g) Parasagital section through the rostral tectum counter-immunostained, as in (d), for ectopic En protein (brown). (h) Parasagital section through E10 tectum, counterstained for RCAS *En-1* transcripts (red), which were detected using a RNA probe labelled with fluorescein isothiocyanate (FITC). Following infection with RCAS *En-1*, both ELF-1 and RAGS become strongly expressed within the rostral tectum. Ectopic En expression within the rostral tectum correlates well with the ectopic expression of both ELF-1 and RAGS. Scale bars: in (b,f,h) = 300 μm; (d,g) = 30 μm.

cell layers (Fig. 4g,h), whereas ELF-1 transcripts were distributed across both deep and superficial cell layers (Fig. 4d). This pattern matches that seen previously for the endogenous expression of RAGS [19] and ELF-1 [20] in the caudal tectum. Similarly, the innermost cell layers also expressed the highest level of ectopic *En-1* mRNA (Fig. 2d) and En protein (Fig. 4d,g). Previous work has shown that at E10, the two deepest layers of the caudal tectum—the stratum griseum periventriculare and stratum griseum centrale — expressed the highest levels of En-2 [15]. Overall, ectopic En expression in the rostral tectum at E10 correlated well with the ectopic expression of both ELF-1 and RAGS. Furthermore, changes in ELF-1 and RAGS expression were specific to the expression of En protein, as no change in the normal RAGS or ELF-1 expression pattern was seen in tecta infected with the control RCAN *En-1* vector (data not shown).

In *Drosophila*, *en* is required during a certain developmental period to positively autoregulate its own transcription [33]. In chick, En expression is induced in host tissue following transplantation of mesencephalic/rhombencephalic tissue [31]. It was therefore of interest to address the question of autoregulation and/or induction in RCAS *En-1*-infected embryos ectopically expressing En. The endogenous expression of *En-1* and *En-2*, however, remained unchanged (as detected using species-specific probes; data not shown), providing further support for the idea that the vertebrate *En* genes are not autoregulated [8,34]. Furthermore, in contrast to the inductive behaviour of grafts, the ectopic expression of mouse *En* alone did not lead to the induction of endogenous chick *En* in adjacent tissue.

### Ectopic En expression alters axonal behaviour

Further evidence suggesting that the rostral tectum has adopted a caudal phenotype was provided by an *in vitro* analysis of retinal neurite behaviour in membrane stripe assays [35,36] (Table 1; Fig. 5). In this assay, when neurites of temporal retinal ganglion cells were given a choice between alternating stripes of rostral and caudal tectal cell membranes, they preferred to extend neurites on membranes from rostral tectum (the natural target of their axons *in vivo*) and were repelled by membranes from caudal tectum (Fig. 5a). In contrast, the neurites of nasal retinal ganglion cells, which normally extend their axons to the caudal tectum, showed no preference (Fig. 5d). We found that when temporal retinal neurites were given the choice of growing on rostral membranes from E9–E10 embryos infected with RCAS *En-1* at E1.5, or on rostral membranes from stage-matched uninfected controls, they preferred the normal rostral membranes and were repelled by RCAS *En-1*-infected rostral membranes (Table 1; Fig. 5b). The degree of the response varied both within and between individual experiments and may have resulted from incomplete and/or varying levels of infection, as discussed below. No preference was seen when temporal retinal neurites were given a choice between rostral tectal membranes from embryos infected with RCAN *En-1* and normal (uninfected) rostral membranes (Table 1; Fig. 5c), showing that the preference was not the result of viral infection and suggesting that the effect was specific to the

expression of En protein. Indeed, immunohistochemical analysis of cell nuclei from the same lysates that yielded membranes of RCAS *En-1*-infected rostral tecta revealed the presence of ectopic En protein (data not shown). Nasal retinal ganglion cell neurites, however, showed little preference for RCAS *En-1*-infected rostral membranes versus uninfected rostral tectal membranes (Fig. 5e).

If the rostral tectum had in fact adopted a caudal phenotype, it might have been expected that temporal retinal neurites would show a more complete preference in our stripe assay. However, the fact that temporal neurites were repelled in 55 %, and not 100 %, of cases ($n = 42$, Table 1) could be explained both by the patchy, and therefore incomplete, transformation of the entire rostral tectum to a caudal phenotype following retroviral infection and/or by variation in the time of onset of RCAS *En-1* expression between different patches. *In vitro* studies by Baier and Bonhoeffer [37] have shown that, when caudal and rostral tectal cell membranes are mixed together and tested against rostral membranes, a growth preference is seen only when the mixing ratio (caudal to rostral) is above a certain threshold (at least one to four). Thus, in our assay, if an insufficient number of cells from the rostral tectum had acquired a caudal phenotype, temporal retinal neurites may not be able to make a clear decision. In addition, transplantation experiments have shown that tectal polarity is labile at E2 and fixed by E3 [26,32,38]. Although our viral infections were made before E2, we cannot assume that every cell that expressed mouse *En-1* at E10 was infected at the time of injection — some cells may have become infected later in development through the lateral spread of the replication-competent vector and may therefore have been only partially transformed or not transformed at all. Thus, differences in both the spatial and temporal expression of En following retroviral infection may contribute toward a rostral-to-caudal transformation that is partial rather than complete. A further consideration is that, in our analysis, cell membrane fractions from several tecta with varying overall levels of infection were combined in each experiment. The ectopic expression of En at E10 — as assayed directly by immunohistochemical analysis of nuclei from rostral RCAS *En-1*-infected tectal cell lysates and/or indirectly by whole-mount RNA *in situ* hybridization analysis of the middle (unused) portion of the tectum — varied from approximately 10 % to greater than 80 % between different individual tecta.

**Table 1**

***In vitro*** **analysis of retinal neurite behaviour in membrane stripe assays.**

| | | Control C/R | | | | Infected R′/R | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 0 | 1 | 2 | *n* | 0 | 1 | 2 | *n* |
| RCAN ***En-1*** | | | | | | | | | |
| Stages 6–8 | T | – | 1R | 8R | 9 | 13 | – | – | 13 |
| | N | 9 | – | – | 9 | 13 | – | – | 13 |
| RCAS ***En-1*** | | | | | | | | | |
| < Stage 7 | T | – | – | 6R | 6 | 4 | 3R | 3R | 10 |
| | N | 6 | – | – | 6 | 4 | – | 4R | 8 |
| Stages 7–8 | T | – | – | 6R | 6 | 11 | 1R | 6R | 20* |
| | N | 6 | – | – | 6 | 16 | – | 1R<br>1R′ | 20* |
| Stages 9–10 | T | – | – | 4R | 4 | 2 | 3R | 3R | 8 |
| | N | 4 | – | – | 4 | 7 | – | 1R | 8 |
| Stage 11 | T | – | – | 2R | 2 | 1 | 2R | 1R | 4 |
| | N | 2 | – | – | 2 | 4 | – | – | 4 |

| | | Summary | | | | | |
|---|---|---|---|---|---|---|---|
| | | Control | | | Infected | | |
| | | ND | D | ***n*** | ND | D | ***n*** |
| RCAS ***En-1*** | T | – | 18R | 18 | 18 | 22R | 42* |
| | N | 18 | – | 18 | 31 | 6R<br>1R′ | 40* |

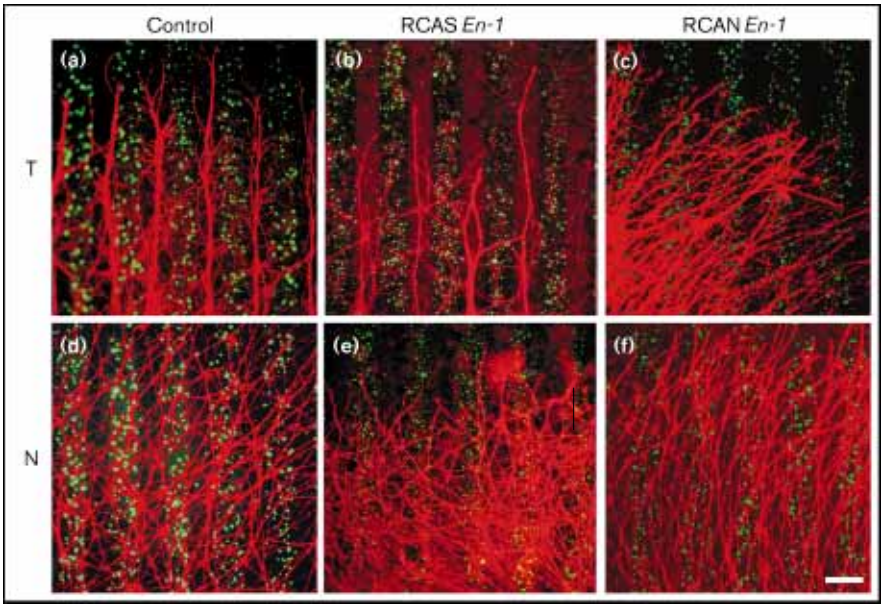
Growth of axons from temporal (T) and nasal (N) retinal ganglion cells was categorized subjectively into 0 (no decision), 1 (moderate response) and 2 (clear decision). Experiments where the control showed no decision or the infection level was low – as judged directly, by immunohistochemical analysis of nuclei from rostral RCAS *En-1*-infected tectal cell lysates, and/or indirectly, by whole-mount RNA *in situ* hybridization analysis of the middle portion of the tectum – were not included in the analysis ($n = 12$). (*No growth was observed on two membrane carpets.) Results for RCAS *En-1* infections were pooled (Summary) to give a total number of responses. C, caudal uninfected cell membranes; R, rostral uninfected cell membranes; R′, rostral RCAS *En-1*- or RCAN *En-1*-infected cell membranes; D, decision; ND, no decision.

### ELF-1 and RAGS are potential downstream targets of En

In chick, the rostrocaudal polarity of the developing tectum is first detectable at E2 by the graded expression of En in the caudal half of the mesencephalic vesicle (reviewed in [22]). Transplantation experiments using chick–quail chimaeras have previously shown that the pattern of En expression correlates well with the subsequent gradient of cytoarchitectonic differentiation [22,25,26,32], as well as with the pattern of retinotectal projections [22,24,38]. Our results provide direct evidence that En expression influences both cytoarchitecture and the expression of molecules thought to be involved in

Although neurites of nasal retinal ganglion cells normally show no preference for rostral versus caudal membranes, we found that they did show a slight preference for uninfected versus RCAS *En-1*-infected rostral membranes (Fig. 5e). Intriguingly, this preference was most obvious for tecta that had been infected with RCAS *En-1* at very early stages (HH stages 5–6; Table 1). That this may have been a result of heavier expression of RAGS by the early-infected tissue is suggested by the observation that nasal (as well as temporal) axons are repelled by RAGS expressed on COS cell membranes [19].

**Figure 5**




Growth of retinal neurites on membrane carpets of infected and uninfected tectal membranes. Typical growth patterns of retinal fibers from the temporal **(a–c)** and nasal **(d–f)** half-retina on various membrane carpets are shown. Axons are growing toward the top. RCAS *En-1*- and RCAN *En-1*-infected rostral membranes and caudal uninfected membranes are marked by the addition of fluorescent beads. (a,d) Normal growth pattern on alternating rostral and caudal membranes from control (uninfected) embryos. (b,e) Growth pattern on alternating rostral tectal membranes from RCAS *En-1*-infected and uninfected embryos. (c,f) Growth pattern on alternating rostral tectal membranes from RCAN *En-1*-infected and uninfected embryos. Scale bar = 100 μm. T, neurites of temporal retinal ganglion cells; N, neurites of nasal retinal ganglion cells.

retinotectal axon guidance, consistent with En playing these roles in its normal domain of expression. Given the strong correlation that has been made between the pattern of En expression and subsequent pattern of retinotectal projections, we would also expect to find alterations in the retinotopic projection map following *En-1* misexpression. Indeed, Itasaki and Nakamura [27] and Friedman and O'Leary [39] have shown that similar overexpression of chick *En* in the tectal primordium disrupts the normal retinotectal projection pattern: nasal retinal axons arborize at ectopic sites in the rostral or middle tectum, whereas most temporal retinal axons fail to innervate the tectum, occasionally degenerating completely. These findings are consistent with — and more importantly can be explained by — our results showing that the rostral tectum has acquired caudal characteristics under the influence of ectopic En expression. En itself though, is not likely to be directly involved in axonal guidance as it is a transcription factor [40,41]. We have shown, however, that two putative axon guidance molecules, RAGS [19] and ELF-1 [20] are both strongly expressed ectopically following misexpression of *En-1* in the rostral tectum. The possibility therefore exists that both RAGS and ELF-1 may be upregulated, either directly or indirectly, by En in their normal domain of expression. Further investigation is required to determine the exact nature of such interactions.

Although neither RAGS nor ELF-1 has yet been shown to act as a guidance molecule *in vivo*, the increased expression of both molecules in the rostral tectum, together with the fact that temporal retinal neurites were preferentially repelled by RCAS *En-1*-infected rostral tectal membranes *in vitro*, support the idea that RAGS and/or ELF-1 might be involved in sorting retinal axons along the rostrocaudal axis *in vivo*. Previous experiments have shown that recombinant RAGS protein produced by COS cells can induce growth cone collapse and repel retinal ganglion cell neurites *in vitro* [19].

### En functions in determining rostrocaudal polarity

Mutational analysis in mice has demonstrated that *En* is required early for normal development of the caudal mesencephalic/rostral rhombencephalic region (reviewed in [11]). Targeted disruption of the *En-1* locus results in the deletion of the midbrain–hindbrain tissue in which it is normally expressed — a phenotype that is apparent as early as E9.5 [7]. Targeted disruption of the *En-2* gene, which is expressed slightly later than *En-1*, results in a much milder phenotype, involving abnormal foliation of the cerebellum [8,9]. Recent analysis has shown that the contrasting phenotypes reflect differences in the temporal and spatial expression of the two mouse En proteins and not a divergence in their biochemical activity [10]. *En-1/En-2* double mutants exhibit a more severe deletion of mesencephalic/rhombencephalic tissues than is apparent in *En-1* mutants [11]. Thus, existing mouse mutants cannot be used to examine the later role of En in the development of midbrain polarity, which was indicated by previous transplantation experiments (reviewed in [22]). By misexpressing En using a retroviral vector in chick, we have been able to demonstrate that En plays a crucial role later in development, in conferring caudal characteristics to developing tectal tissue. This strongly suggests that En normally plays this role within its endogenous expression domain, the caudal mesencephalon. Our experiments

show that under the influence of ectopic En, at high levels of expression, rostral tectum can adopt a very caudal phenotype. This approach, however, cannot directly address the question as to how the normal graded expression of En leads to corresponding gradients of cytoarchitecture and retinotopic projections, where precise control of transcription would be required.

## Conclusions

Here, we have shown that the rostral tectum adopts a caudal phenotype following RCAS *En-1* infection at early embryonic stages. The gradient of cytoarchitectonic differentiation is abolished, and two molecular markers that are normally expressed in a decreasing caudal-to-rostral gradient in the tectum are strongly expressed rostrally. In addition, cell membranes from rostral tectum of RCAS *En-1*-infected embryos preferentially repel temporal axons in *in vitro* membrane stripe assays. A major challenge will now be to determine the molecular mechanisms through which *En* acts to confer positional specification on cells along the rostrocaudal axis of the tectum; such analyses should provide valuable insight into the formation of topographically ordered retinotectal projections.

## Materials and methods

### In situ *hybridization and immunohistochemistry*

Whole-mount RNA *in situ* hybridization of intact embryos or partially dissected embryonic brains was performed using non-radioactive RNA probes labelled with digoxygenin (DIG) or FITC (see below) as previously described [42], except that following the first post-hybridization wash, the embryos were rinsed three times for 10 min with 0.5 M NaCl, 10 mM Tris–HCl (pH 7.5) and 0.1 % Tween-20, and then treated for 1 h at 37 °C with 100 μg $ml^{-1}$ RNase A in the same buffer. Samples were refixed in 3.5 % paraformaldehyde, before sectioning, and/or in methanol:DMSO (4:1), before subsequent immunohistochemical analysis.

En protein was detected by whole-mount immunohistochemistry using the polyclonal antiserum αEnhb-1 (kindly provided by A. Joyner) as previously described [5], except that samples were incubated in a 1:1000 dilution of crude antiserum for 3–5 days. Samples were refixed in 3.5 % paraformaldehyde before sectioning.

### *Probes*

Antisense DIG- or FITC-labelled RNA probes were synthesized according to the manufacturer's instructions (Boehringer Mannheim, Indianapolis, Indiana) using the following appropriately linearized DNA templates: for *En-1*, a previously isolated ~800 bp *Bgl*II–*Sst*I chick genomic DNA fragment extending 3′ from the homeobox [29] was subcloned into a Bluescript vector (Stratagene, La Jolla, California), and the resulting plasmid linearized using *Eco*RI. For *En-2*, a similar ~1.2 kb *Bgl*II–*Sma*I chick genomic DNA fragment [29] was subcloned and subsequently linearized using *Xba*I. For RAGS, a 328 bp probe covering the 3′ portion of a previously isolated cDNA [19] was made following *Pvu*II digestion of the full-length chick cDNA clone. For ELF-1, a 910 bp chick cDNA clone that contains the entire coding region was obtained by screening a chick E8 posterior tectum cDNA library with a PCR-generated 610 bp probe for the region encoding mouse ELF-1 [43], and subcloned into Bluescript II $KS^+$ from which the multiple-cloning site had been deleted with *Apa*I and *Sac*II. The subcloned full-length chick cDNA was linearized by cutting internally with *Pst*I and transcribed to yield a 321 bp probe that included 70 bp of the coding region and 251 bp of the 3′ untranslated region. To detect retroviral transcripts containing mouse *En-1* cDNA sequences, a 176 bp *Sst*I–*Apa*I fragment from a previously isolated cDNA [30] was subcloned into a Bluescript vector and the resulting plasmid linearized using *Sst*I.

### *Wax and vibratome sectioning following whole-mount RNA* in situ *hybridization and immunohistochemistry*

Selected embryos or partially dissected embryonic brains were embedded in gelatin/albumin, and 50–100 μm serial sections were cut along the sagittal plane using a vibratome. Sections were then cleared in 80 % glycerol in PBS and mounted under coverslips for photography. Alternatively, samples were dehydrated in a graded series of ethanol concentrations and embedded in paraffin. Similar serial sections of 10 μm thickness were then cut, stained using cresyl violet, and mounted under coverslips for photography.

### *Retroviral construction and infection*

The entire coding region plus the first 19 bp of the 3′ untranslated region of the mouse *En-1* cDNA [29,30] was subcloned in frame into the Cla12Nco adapter plasmid [28] using appropriate restriction sites within the polylinker. A *Cla*I fragment of ~1.3 kb was then purified following partial digestion of this vector with *Cla*I and subcloned into the retroviral vectors RCAS(BP)A and RCAN(BP)A [28] generating RCAS *En-1* and RCAN *En-1*, respectively. RCAN(BP)A is a variant of RCAS(BP)A from which the splice acceptor immediately upstream of the *Cla*I site has been removed, preventing translation of the inserted gene and acting as a control for non-specific effects resulting from viral infection.

Chick embryo fibroblasts and concentrated viral stocks were prepared as previously described by Fekete and Cepko [44]. Viral titers ranged from $\sim 2 \times 10^8$ to $10^9$. Concentrated viral supernatant was either injected into the lumen of the developing neural tube of HH stages 8–11 (E2) embryos as described [44], or, for injections carried out before HH stage 8, layered on top of the anterior neuroepithelium or over the neural groove. All experimental manipulations were performed using white Leghorn chick embryos provided by Poyndon Farm (Hertfordshire, UK).

### In vitro *membrane stripe assay*

*In vitro* membrane stripe assays were performed essentially as described by Baier and Klostermann [45]. Optic tecta were dissected from E9–E10 chick embryos. Tectal pieces from between two and ten embryos were pooled in the preparation of various membrane fractions.

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