# Hindbrain patterning revisited: timing and effects of retinoic acid signalling

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**Summary**

**Retinoids play a critical role in patterning, segmentation, and neurogenesis of the posterior hindbrain and it has been proposed that they act as a posteriorising signal during hindbrain development. Until now, direct evidence that endogenous retinoid signalling acts through a gradient to specify cell fates along the anteroposterior axis has been missing. Two recent studies tested the requirement for retinoid signalling in the developing hindbrain through systematic application of a pan-retinoic acid receptor antagonist.[1,2] They demonstrate a stage-dependent requirement for increasing retinoid signalling activity along the hindbrain that proceeds from anterior to posterior. Together these findings challenge the concept of a stable gradient of retinoic acid across the hindbrain and warrant a re-interpretation of the phenotypes obtained by genetic and nutritional disruption of retinoid signalling in the amniote embryo. *BioEssays* 23:981–986, 2001. © 2001 John Wiley & Sons, Inc.**

## Introduction

Classic models for the patterning of the central nervous system (CNS) propose that the anteroposterior axial pattern is established by different anterior- and posterior-inducing signals in the gastrula. One hypothesis postulates that anterior identity is induced by early invaginating endomesodermal tissues, while posterior fates are specified by late invaginating mesoderm.[3] An alternative model, which has won much support since it was first suggested by Nieuwkoop and colleagues, proposes a two-step mechanism that regionalises the developing neuroectoderm: an activating signal establishes neural differentiation towards an anterior fate, and a subsequent transformation signal elaborates on this pattern and posteriorises the CNS to form hindbrain and spinal cord. This transforming activity is present as a gradient during gastrulation and neurula stages, with low levels inducing hindbrain fates and high levels inducing spinal cord fates.[4,5]

Retinoic acid (RA) has been a strong contender for the transforming factor,[6] as it fulfils several of the required criteria. When developing embryos are exposed to RA, forebrain and anterior neural markers are reduced and hindbrain and spinal cord fates are expanded anteriorly, resulting in a posteriorisation of the CNS. Furthermore, high concentrations of endogenous RA have been detected in the trunk and tail of vertebrate embryos, with a sharp anterior boundary at the level of the posterior hindbrain.[7] Following these promising early efforts to unravel the function of RA in CNS patterning, it has subsequently been difficult to test its actual role during development. The ability to interfere with RA signalling in vivo, however, has rekindled interest in RA and allowed the hypothesis that endogenous RA functions as a posteriorising factor to be tested.

Since RA is synthesised from vitamin A, it is possible to investigate the effects of reduced RA signalling by depriving embryos of dietary sources of vitamin A (Fig. 1). This causes a variety of developmental defects, collectively known as fetal vitamin A-deficiency (VAD) syndrome.[8,9] As expected if RA had transforming activity, a disruption of hindbrain patterning is a typical feature of VAD in amniotes.[10–13] Specifically, the posterior hindbrain in VAD quail embryos appears to be transformed towards more anterior fates, resulting in enlarged anterior rhombomeres (r) and a truncation of rhombomeres posterior to r3.[10] As described below, it is now evident that cells in the posterior hindbrain are respecified towards more anterior fates.[14] RA can rescue this defect in a dose-dependent manner, as shown through the simultaneous application of defined concentrations of RA to VAD rat embryos, which results in a gradual loss of posterior rhombomeres with decreasing amounts of RA.[13]

The conversion of retinaldehyde, the oxidative product of vitamin A, to RA is catalysed by retinaldehyde dehydrogenase 2 (Raldh2) (Fig. 1).[15,16] Because mutations in *Raldh2* in the mouse mimic the defects of VAD syndrome and its expression in the paraxial mesoderm and in the somites correlates with the distribution of endogenous RA in chick and mouse embryos, Raldh2 is thought to be the main source of RA produced during gastrulation and somitogenesis stages.[17,18] One prediction of the activation-transformation model is the existence of a gradient of transforming activity across the hindbrain. The expression of RA-metabolising enzymes and the concentration-dependent response of hindbrain cells to RA imply the existence of a graded signal. Raldh2 provides a source of RA at the posterior end of the hindbrain, while expression of *Cyp26* in the anterior neuroectoderm, a cytochrome P450 that inactivates RA,[19] could serve as a sink that keeps the

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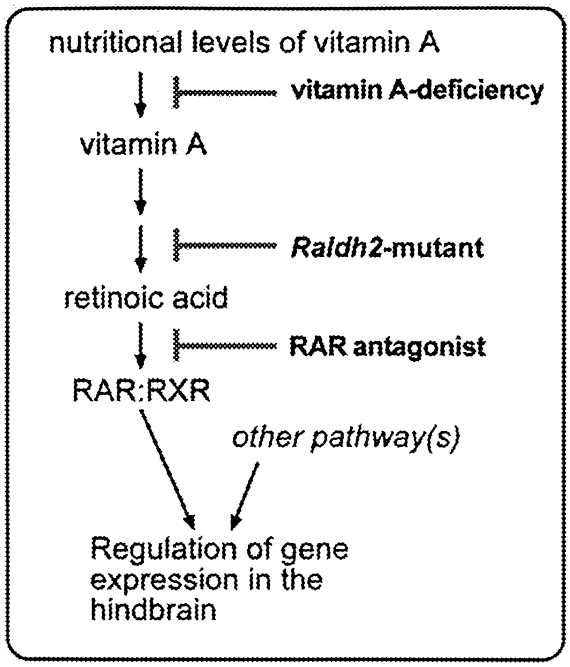


**Figure 1.** Approaches to inhibiting retinoic acid mediated gene regulation in the developing hindbrain. The supply of retinoic acid to the developing embryo can be restricted by depletion of nutritional sources of vitamin A. Subsequent biosynthesis of retinoic acid (RA) involves Retinaldehyde dehydrogenase 2 (Raldh2). Retinoic acid binds to and activates nuclear retinoic acid receptors (RARs), which form heterodimers with retinoid "X" receptors (RXRs) to regulate the expression of target genes. Downstream of vitamin A uptake, the signalling cascade leading to RAR activation is blocked in *Raldh2* mutants, while activation of RAR signalling can be inhibited by RAR-specific antagonists.

anterior hindbrain free of RA and sharpens the resultant gradient.[20–23] Efforts to detect a gradient of RA across the hindbrain have failed, however, raising the possibility that RA-mediated signalling may depend on regulatory mechanisms that are more complex than a simple stable concentration gradient of RA.

RA modulates gene transcription by binding to and activating retinoic acid receptors (RARs). Three such receptors have been identified, RAR$\alpha$, -$\beta$ and -$\gamma$, and are thought to form heterodimers with the retinoid X receptors (RXR$\alpha$, -$\beta$ and -$\gamma$) (Fig. 1).[24] The genes encoding the murine RARs have been knocked out in the past, but inactivating a single gene at a time only recapitulates some phenotypic aspects of full VAD, supporting the idea that RARs have partially redundant functions.

The classical non-conditional gene knockout approach is not particularly well suited to dissect the requirements of the RARs over time. For instance, it is not known either where the proposed gradient of endogenous RA signalling is active along the hindbrain, or when retinoids exert their transforming activity. While conditional gene knockouts may provide an answer to these questions, finer control over timing is achieved using the reversible inactivation of RARs through antagonistic compounds (Fig. 1). These antagonists competitively inhibit the activation of RARs by potent activating agonists and are thus believed to directly interfere with RAR activation. Antagonist-based studies have been performed in the past to dissect retinoid function in limbs and pharyngeal arches,[25,26] yet only now have they been applied to probe the exact requirement of RA in hindbrain development.[1,2]

## Timing of retinoic acid requirement in hindbrain patterning

Dupé and Lumsden[1] studied the consequences of reducing RA signalling for hindbrain segmentation in the chick embryo. In order to determine the developmental stages sensitive to reduced retinoid signalling, an antagonist dose that blocks all RAR-mediated signalling from a given developmental stage onwards was systematically applied. The most severe defects in rhombomere formation are observed when inhibition commences at the end of gastrulation. The effects of antagonist treatment include a posterior enlargement of r3, and the replacement of the typical segment-restricted expression of *Hoxb1* in r4 with a continuous expression domain starting anterior to the level of the first somite and extending to the posterior end of the neural tube. The boundary between r3 and r4 is ill defined in treated embryos, such that cells expressing markers of either rhombomere identity are interspersed. Moreover, *MafB/Kreisler*, a marker for r5 and r6, is not expressed in antagonist-treated embryos, a phenotype strikingly similar to *Raldh2*-null mutants and complete VAD, where posterior rhombomeres are deleted at the expense of an enlarged r3 and r4 (Fig. 2A,C).

When treatment commences at later stages, r3/4 boundary formation proceeds normally, while more posterior rhombomeres are expanded and possess patchy boundaries. Therefore, the later that signalling through RARs is inhibited, the more gene expression boundaries are established correctly and, after the 11-somite stage, when hindbrain segmentation becomes morphologically discernible, RA signalling is no longer required for hindbrain patterning. Thus the formation and subsequent sharpening of gene-expression boundaries in future rhombomeres requires a transient retinoid signal, which appears to shift gradually in an anterior-to-posterior sequence and precedes visible boundary formation by several hours.

## Retinoic acid acts in a concentration-dependent manner

These results imply that the segmentation process requires stage-dependent RA signalling, but they do not directly

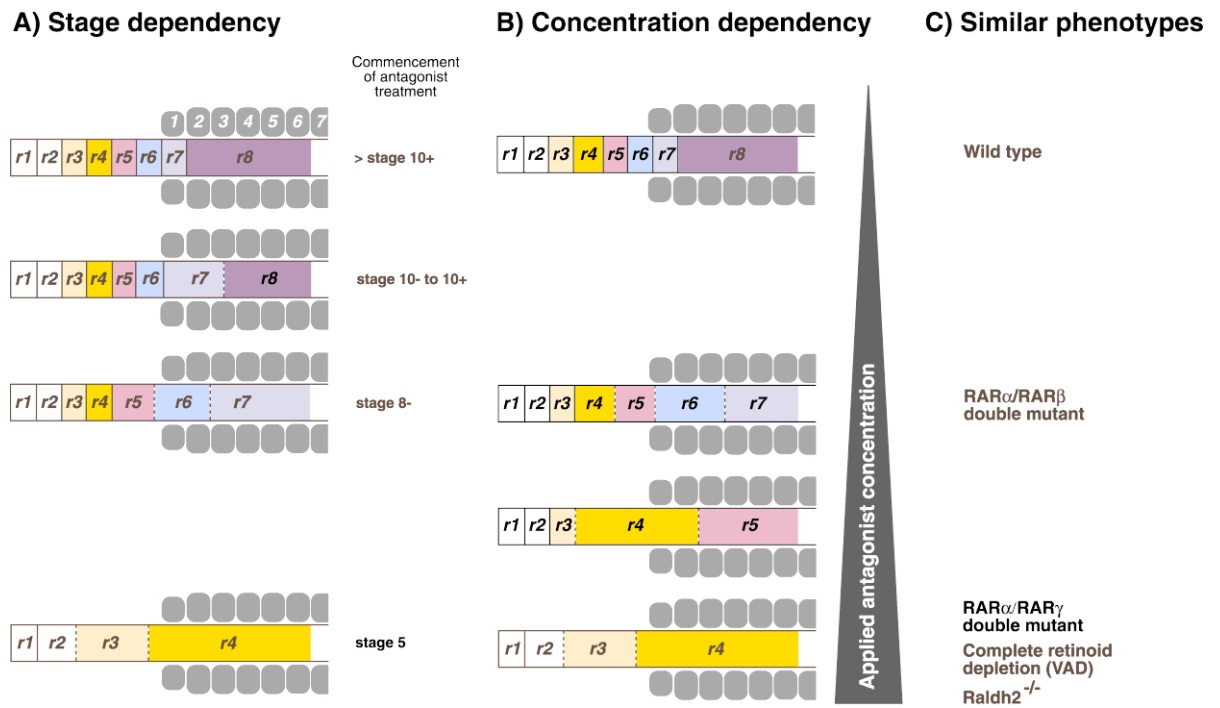


**Figure 2.** Hindbrain patterning is regulated by stage-dependent responses to threshold concentrations of retinoid signalling. Idealised schematic representation of the developmental defects generated in the chick hindbrain through treatment with a pan-retinoic acid receptor antagonist. Identical phenotypes are found when signalling is inhibited from different stages of development onwards, when overall signalling is reduced throughout a signal-dependent phase and upon genetic and nutritional disruption of retinoid signalling in amniote embryos. **A:** The absence of retinoid acid (RA) signalling from a given developmental stage onwards demonstrates that the signalling requirement is stage-dependent. Early onset of treatment (stage 5)[37] results in the development of only four rhombomeres with posteriorly shifted expression boundaries; posterior rhombomeres can develop and are enlarged when treatment starts later. RA signalling is required for hindbrain patterning between the head process (stage 5) and eleven somite stage (stage 10+). **B:** Reduced RA signalling through increasing concentrations of antagonist (indicated on the right by concentration bar) results in phenotypes that are comparable to stage dependent inactivation. Thus lower levels of activity are required at earlier stages and will activate a genetic programme typical of more anterior rhombomeres. **C:** Combinations of *RAR*-knockouts, mutants in *Raldh2*, and complete vitamin A-deficiencies all compare to phenotypes generated by intermediate or absent RA signalling. Comparable phenotypes for all panels are represented on the same horizontal level. Invariable hindbrain boundaries are depicted as solid lines; experimental variation in the anteroposterior extent of boundary formation and fuzzy boundaries are depicted as stippled lines, respectively. Gene expression in pre-rhombomeres (r) is indicated; the first seven somites are indicated as grey boxes.

address the more fundamental question of whether or not endogenous RAR activity forms a gradient along the antero-posterior extent of the hindbrain. What are the consequences for boundary formation when RAR activity is continuously antagonised throughout the signalling-sensitive phase? Interestingly, the phenotypes obtained with various concentrations of RAR antagonist are very similar to those obtained in the previous experiment (Fig. 2B). For example, anterior rhombomeres r3 and r4 still form but their posterior boundaries shift towards the posterior with complete abolishment of RA signalling, suggesting that RAR activity is not required to specify r3 and r4 territories as such. However, some RA signalling is required to refine their anterior and posterior limits. In contrast, the formation of r5–r8 seems to be absolutely dependent on RA signalling and is most sensitive to changes in retinoid levels, such that even low concentrations of antagonist have profound effects on rhombomere formation and positioning.

It appears that hindbrain precursor cells respond to a gradient of RA signalling strength by establishing rhombomere-specific gene expression patterns according to the local intensity of the signal. Low levels of RA signalling appear to be necessary only for the refinement of gene expression boundaries in the anterior hindbrain, while the specification of posterior rhombomeres (r5–r8) requires a higher activation threshold, both for the initialisation as well as refinement of posterior expression boundaries. An overall reduction of signalling would thus cause a posterior expansion of pre-

rhombomeric expression boundaries and a failure of posterior hindbrain specification.

### An emerging general mechanism for amniote hindbrain patterning

Dupé and Lumsden[1] further noted that the posterior-most gene expression domain in the hindbrain in antagonist-treated embryos is expanded into the postotic neural tube, up to the level of the sixth somite. Because ectopic expression of anterior rhombomere markers never extends posterior to this apparent boundary, they argue that this region should be considered the posterior-most part of the hindbrain, r8. This finding warrants a second look at the interpretation of the phenotypes of VAD in amniote embryos. Because the distinctive stripe of *Hoxb1* expression in r4 is absent, the posterior boundary of r3 in VAD quail embryos appears to abut the spinal cord, where *Hoxb1* is also expressed. This was interpreted as a loss of r4,[10] however an alternative explanation is that the entire posterior hindbrain is composed of an enlarged r4. This is also consistent with the posterior expansion of r4-specific markers in VAD rat embryos and the *Raldh2*-null mouse.[13,18] Together, this suggests that the hindbrain region of the neuroectoderm extends further posteriorly than previously thought and that this region can be transformed into an r4-like identity with loss of RA signalling.

In a second study, Wendling et al.[2] have further resolved the RAR-knockout phenotypes in mice, which previously had been difficult to interpret due to the temporal and spatial dynamics of RAR expression. Application of the pan-RAR antagonist to the mouse embryo results in similar phenotypes to those in the chick: application from the head process stage onwards results in a posterior expansion of r3 and r4 territories and loss of r5 and r6, whereas later treatment at the 2 to 4-somite stage expands r5 and r6 posteriorly. Interestingly, both situations phenocopy the defects in *RARα/RARγ* double-mutant mice, whose gene expression patterns in the hindbrain are almost identical to those in VAD and *Raldh2*-null embryos. All these conditions are likely to represent a state of complete RA deficiency and substantiate the hypothesis that Raldh2 is the only source for RA in the hindbrain.[18] In comparison, the compound mutation of *RARα/RARβ*[27] exhibits a posterior expansion of r5 and r6 (Fig. 2C) and phenocopies the defects observed when RA signalling is either partially reduced or eliminated at later stages, from the 2–4 somite stage onwards. In this light it seems likely that *RARα* and/or *RARγ* mediate RA signalling in early stages of hindbrain segmentation by regulating genes within the posterior hindbrain, while *RARβ* is required at a later stage to specify the posterior boundary of r5 and r6, corresponding to its expression in the posterior neural tube up to the r5/6 boundary in mouse and chick at this stage. Intricate regulatory dependencies, including the direct regulation of *RAR* expression by RA, are certain to be involved in this process and may account for some of the differences between *Raldh2*-null embryos and *RARα/RARγ* double mutants.[2]

Taken together, these two studies indicate a conserved requirement for RA signalling in mice and chick at similar stages and suggest which RAR subtypes may be involved at different stages of hindbrain patterning.

### Somites as a source of posteriorising signals

These results not only emphasise the importance of endogenous retinoid signalling, but they elegantly demonstrate that hindbrain segmentation is sensitive to different threshold levels of RA along the anteroposterior axis. While this lends support to the theory that a gradient of RA-mediated signalling establishes rhombomeric boundaries through the posteriorisation of the hindbrain,[20,28–30] the composition of the gradient and its regulation remain enigmatic. Although the requirement for RA signalling in the determination of hindbrain gene expression boundaries is limited to a particular period of time, the mechanisms involved allow for unpredicted flexibility, because both exogenous application and injection of RA into the hindbrain can rescue rhombomere formation in RA-signalling-deficient embryos even when applied long after the RA-sensitive stages for the correct establishment of gene expression domains.[13,14,18] Under these conditions, RA cannot form a gradient itself, therefore this finding is difficult to reconcile with an invariable RA signalling gradient that is maintained throughout the RA-sensitive period in the hindbrain.

The expression of *Raldh2* in paraxial mesoderm during the signalling-sensitive phase[23,30,31] and the ability of somites to mimic the posteriorising effects of RA by modifying gene expression in the hindbrain[32] have implicated the somites as the source of the RA signal required for hindbrain segmentation. More recently, the requirement for somitic *Raldh2* expression to posteriorise the postotic hindbrain has been demonstrated directly.[33] Accordingly, the increase of embryonic RA production during somitogenesis corresponds to the upregulation of somitic *Raldh2* expression. It would seem safe to assume that RA signalling is equally activated by all anteroposterior levels of the somitic mesoderm. When single somites are transplanted anteriorly to the preotic region, however, they can induce gene expression that is characteristic of high levels of RA signalling. This posteriorising activity is a transient feature within the first 5–6 somites and is lost progressively in an anterior-to-posterior manner.[32] This suggests an additional level of regulation, so that not only the reception of the signal by the pre-rhombomeric region, but also signalling itself is subject to continuous change during the course of hindbrain patterning.

Interestingly, there are further similarities between somite signalling and the requirement for RA signalling in the hindbrain: posterior regression of the signalling activity of mesoderm ceases around the same stage of development

(11-somite stage) as the RA-signalling-sensitive period of hindbrain segmentation, and never extends beyond the region of somites 4 or 5, coinciding with the caudal limit of gene expression in r8.[32] Thus the caudal regression of posteriorising activities could be involved in the modulation of a dynamic gradient of RA-signalling activity in the posterior hindbrain. This mechanism could further account for the fact that the postotic hindbrain, which is in contact with anterior somites and therefore would seem to be exposed to overall high levels of RA signalling, will give rise to different rhombomeres under situations of reduced RA signalling (Fig. 2B).

The mechanisms that modify RA signalling activity are presumably complex and may involve several levels of regulation. For example, additional activities like those mediated by Cyp26 may exist that inactivate RA, and the production, diffusion or local activity of RA may be modulated by still unknown factors. Some of these additional factors are likely to be identified within the paraxial mesoderm and its derivatives, which have been recognised in previous studies as a source of additional posteriorising activities that may operate as a chaperone for RA or may act in a parallel pathway.[34,35] Fibroblast growth factors are good candidates, as they have been shown to participate in regulating *Krox-20* and *MafB/Kreisler* expression in r5 and r6.[36] It remains to be uncovered if RA itself is distributed in a gradient and if long-range diffusion of RA actually takes place. Identifying the nature and sources of factors that participate in modifying RA signalling and the response of the hindbrain will present a challenge for the future.

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