Anterior-posterior axis formation in Xenopus laevis

Jansen, H.J.

CHAPTER 4

Retinoid signalling is required for information transfer from mesoderm to neuroectoderm during gastrulation.

F. Lloret Vilaspasa, H.J. Jansen, K. de Roos, R. Chandraratna, M.H. Zile, C.D. Stern and A.J. Durston

Submitted

Abstract

The hindbrain region of the vertebrate central nervous system (CNS) presents a complex regionalisation. It consists of 7-8 distinct morphological segments called rhombomeres, each with a unique identity provided by combinations of transcription factors. One class of signalling molecules, retinoids, have been shown to be crucial for hindbrain patterning through direct trans-activation of *Hox* genes in the neuroectoderm. However, how this morphogen acts is not yet fully understood. Here, we show that the retinoid receptor antagonist AGN193109 causes a posterior hindbrain defect in *Xenopus*, comparable to that seen in other vertebrates. We showed that this defect arises during gastrulation. Blocking endogenous retinoid activity during gastrulation causes downregulation of the most 3' *Hox* genes (paralogues 1-5) in gastrula neuroectoderm, but their initial activation in gastrula non-organiser mesoderm is unaffected. Similar results were obtained in avian embryos: Vitamin A-deficient quail embryos have defective expression of 3' *Hox* genes (i.e. Hoxb1, Hoxb4) in the neural tube, but their early expression in the primitive streak and emerging paraxial and lateral mesoderm, is not affected. In Xenopus, depletion of retinoids from mesoderm by targeted injection of mRNAs for the retinoic acid catabolising enzyme xCYP26 and the cellular retinoic acid binding protein xCRABP blocks 3' *Hox* gene expression in the overlying neuroectoderm. We propose that gastrula's non-organiser mesoderm and its later derivative, the paraxial mesoderm, is the source of a retinoid, which acts as a "transforming" (caudalising) signal for the future posterior hindbrain.

Introduction

There is much evidence that signalling via active retinoids (vitamin A metabolites) is important for early patterning events during development of the vertebrate central nervous system (CNS) (reviewed in (Durston et al., 1998)). Vertebrate embryos go through a sensitive period, starting during gastrulation, when the developing CNS is drastically posteriorised by exposure to the active retinoid all-trans-retinoic acid (RA) (Avantaggiato et al., 1996; Durston et al., 1989; Sive et al., 1990). This agent mimics the action of an endogenous intercellular signal which patterns the developing CNS at this stage (Doniach, 1995; Durston et al., 1998; Lumsden and Krumlauf, 1996a). Key regulatory genes involved in CNS patterning are transactivated directly in neuroectoderm by specific binding of retinoic acid receptor (RAR) and retinoid receptor (RXR) heterodimers to retinoic acid responsive elements (RAREs) (Marshall et al., 1996). Among the retinoid targets are the *Hox* genes, which are crucial for patterning the posterior CNS (hindbrain and spinal cord). Retinoid-regulated *Hox* genes are situated 3' in *Hox* clusters (Durston et al., 1998; Gavalas and Krumlauf, 2000; Lumsden and Krumlauf, 1996b). Members of *Hox* paralogue groups (pg) 1-5 are activated by ectopic RA, while members of pg 6-9 are not (Bel-Vialar et al., 2002; Godsave et al., 1998a).

To identify developmental functions of retinoids, one approach is to examine the consequences of blocking retinoid signalling. This has been possible by a variety of approaches in different vertebrates, for example: vitamin A starvation in quails (Maden et al., 1996), blocking RAR/RXR transactivation by ectopic expression of dominant negative RAR receptors in *Xenopus* (Kolm et al., 1997; Blumberg et al., 1997; van der Wees et al., 1998), blocking synthesis of active retinoids by mutation of the mouse gene for the enzyme retinal dehydrogenase 2 (*Raldh2*) (Niederreither et al., 1999; Niederreither et al., 2000) or mutation of the zebrafish *Raldh2* gene (Begemann at al., 2001; Grandel et al., 2002), overexpression of the

RA catabolising enzyme CYP26 in Xenopus and zebrafish (Hollemann et al., 1998; Kudoh et al., 2002) and applying RAR/RXR synthetic inactive ligands that competitively prevent normal RA binding in chick and zebrafish (Dupe and Lumsden, 2001; Hernandez et al., 2007). All of these studies uncovered a common phenotype caused by loss of retinoid signalling in early vertebrate embryos: lack of segmentation of the posterior hindbrain (rhombomeres (r) 5-8) and transformation of this tissue into more anterior hindbrain. Therefore retinoid signalling is essential for patterning the posterior hindbrain.

A conserved set of retiniod synthesis pathway genes have been implicated directly in laying down the basic organisation of the vertebrate hindbrain. Among these are genes for vitamin A metabolic enzymes (Hernandez et al., 2001), as well as a network of transcription factors that appear to set up the boundaries between, and the identities of, different rhombomeres (Vesque et al., 1996; Helmbacher et al., 1998, Theil et al., 1998). *Hox* genes and their collinear expression appear to have a prominent role in hindbrain regionalisation. They (at least the most 3' genes of each cluster) are expressed very early in development, in the dorsal neuroectoderm of the gastrula, preceding other genes that have been implicated in hindbrain patterning. During this early activation, *Hox* transcripts are detectable not only in the neuroectoderm but also and even earlier, in the mesoderm. Surprisingly, little attention has been given to this initial phase of *Hox* expression and much less to hox gene expression in the early gastrula non-organiser mesoderm. Most work to date has concentrated on the later role of *Hox* genes in patterning the hindbrain, while the mesoderm itself has only been studied later, as a source of signals that pattern the overlying hindbrain: heterotopic grafts in avian embryos demonstrated that morphogens emanating from the somites (including RA) are needed to set up the right pattern in the adjacent rhombomeres (Itasaki et al., 1996; Gould et al., 1998). Nevertheless, these experiments were performed long after gastrulation, during somite stages and it has not been investigated whether the early activation of *Hox* genes or retinoid signalling from the early gastrula non-organiser mesoderm play a role in hindbrain patterning.

On the other hand, it is also important to reveal the inductive events that lead to appropriate *Hox* expression in the early mesoderm. Indeed, misexpression of some Hox genes brings about homeotic transformations in mesodermal derivatives, such as changes in vertebral identity (Ramirez-Solis et al., 1993; Rijli et al., 1995; Subramanian et al., 1995). Interestingly, it was recently shown by means of mutations in regulatory regions of both *Hoxa-10* and *Hoxa-11* that these genes specify the identity of the corresponding vertebrae by their expression in the presomitic mesoderm, rather than in the somites that derive from it (Carapuço et al., 2005), emphasising the importance of the earliest phase of *Hox* expression in the mesoderm.

Here we blocked retinoid function during early development of *Xenopus laevis*. The synthetic retinoid AGN193109 (AGN) has been characterised as being a high affinity antagonist for all three RAR receptor subtypes (Agarwal et al., 1996). We find that when added to *Xenopus* embryos during gastrulation, AGN causes a mid-axial/hindbrain phenotype: from neurula stages, this resembles the posterior hindbrain phenotypes previously obtained by other approaches (see above), confirming the effectiveness of this reagent for abrogating retinoid signalling. We then investigate the origin of this mid-axial defect in the CNS during *Xenopus* development and find that AGN does not affect the very earliest *Hox* gene expression in (non-organiser) mesoderm. Vitamin A-deficient (VAD) quail embryos (deprived of maternal supply of vitamin A and therefore unable to synthesise retinoids) display a similar phenotype. Towards the end of gastrulation, AGN treatment causes loss of expression of mid-axial genes in neuroectoderm. At this stage and in the early neurula, the defect is more extensive than that

seen later, at the late neurula stage; by then, the early phenotype is partly repaired, resulting in a more localised defect.
These results indicate that retinoids are involved in mediating information transfer from mesoderm to neuroectoderm during gastrulation, perhaps corresponding to the transformation signal in Nieuwkoop's activation-transformation model of CNS patterning (Nieuwkoop, 1952). To test this we blocked retinoid function in gastrula mesoderm by ectopic expression in mesodermal cells (but not neuroectodermal cells) of the RA catabolic enzyme xCYP26 and the RA binding protein xCRABP. This blocks neuroectodermal expression of hindbrain (3') *Hox* genes, supporting the idea that retinoid function in mesoderm is required for patterning of the adjacent neuroectoderm.
We propose that in *Xenopus* the identity of the posterior hindbrain (r4-8) is acquired during gastrulation, that retinoids are necessary for the correct patterning of this region of the CNS by regulating the expression of *Hox* genes in specific rhombomeres (pg 1-5) and that earlier expression of the same genes in the underlying non-organiser mesoderm is not affected by retinoid loss of function. Thus, initiation of Hox expression, which takes place in the non-organiser mesoderm during early gastrulation, is RA-independent. We therefore suggest that RA acts as a vertical signal from this mesoderm to instruct the overlying prospective hindbrain region.

Results

The general retinoid antagonist AGN193109 impairs retinoid signalling

To assess the effect of the RAR antagonist AGN on retinoid signalling in early *Xenopus*, embryos were injected with a DR-5-TATA-luciferase reporter and the embryos then treated with 10^{-6} M AGN or 10^{-6} M RA during gastrulation. Luciferase activity after antagonist treatment decreased to 30% of that measured in untreated or carrier (0.1% DMSO) treated embryos, whereas it increased nearly 4-fold after treatment with 10^{-6} M RA (see Figure 7 in Supplementary data). These results show that AGN impairs retinoid signalling in vivo.
We investigated if AGN can inhibit teratogenicity of exogenously applied RA (Fig. 1A). As previously described, treatment of gastrula stage embryos with 10^{-6} M RA causes severe anterior truncations, lost or reduced eyes and a reduced cement gland (Durston et al., 1989; Sive et al., 1990). However, co-application of 10^{-6} M RA with 10^{-6} M AGN rescues the development of eye pigment and cement gland, similar to embryos treated with AGN only; whereas co-application of 10^{-7}M AGN with 10^{-6}M RA yielded an intermediate rescue. These results show that AGN can antagonise RA teratogenicity. Furthermore, treatments with 10^{-7}M AGN or 10^{-6}M alone caused increasing shortening of the A-P axis.

Retinoid loss of function by the general retinoid antagonist AGN generates mid-axial defects

The luciferase assay above showed that treatment with 10^{-6} M AGN not only interferes with the effects of exogenously applied RA, but also causes a decrease in endogenous retinoid signalling. This predicts that AGN treatment should also cause an axial patterning phenotype similar to those previously observed using diverse RA loss of function approaches. We used immunohistochemistry with the antineural antibodies 2G9 (Jones and Woodland, 1989a) and Xen-1 (Ruiz I Altaba, 1992a) and confocal microscopy to analyse changes in the morphology of the tadpole (st. 45) CNS caused by AGN treatment. Examination of the brain revealed a compressed prosencephalon and mesencephalon. The anterior hindbrain is slightly enlarged and properly segmented, rhombomeres 4 and 5 are

quite normal in size but less distinctly segmented, whereas rhombomeres 6, 7 and 8 are truncated or absent (Fig. 1B). These findings confirm that AGN causes posterior hindbrain defects similar to those seen using other methods to deplete retinoid signalling.

The definitive hindbrain defect caused by AGN treatment is established by the late neurula

We examined the developmental changes in the patterning of the CNS caused by blocking retinoid signalling. By the end of neurulation (st. 20), we observed the following effects of AGN (Fig. 2A-G): the *Hoxb-1* anterior domain, normally restricted to the prospective r4, expands towards the future spinal cord (Fig. 2A). In contrast, the *Hoxd-3* expression domain (r5-6) is reduced as compared to control embryos (Fig. 2B). *Hoxb*-4 expression becomes undetectable in the presumptive hindbrain and the distance between the *En-2* stripe at the mid-/hindbrain boundary and the anterior *Krox-20* stripe (r3) increases, indicating an enlargement of r1-2 (Fig. 2C). In control embryos, *Hoxa-5* is expressed in two stripes in the future anterior spinal cord and posterior hindbrain; both stripes disappear after AGN treatment (Fig. 2D). On the other hand, retinoid depletion expands the normal *Hoxc-6* expression domain anteriorly (prospective spinal cord), but its intensity appears unaffected (Fig. 2E). Expression of *Otx*-2 *and Xcad3* (markers of fore- midbrain and posterior spinal cord domains, respectively) suggests a slight reduction of the overall hindbrain length as compared to normal embryos (Fig. 2F). Expression of *XlPOU2* (r2; Fig. 2G) and *Krox-20* (r3/r5; Fig. 2C,E) show an enlarged anterior hindbrain (r1-3); the gap of *XlPOU2* expression between prospective r2 and r4 is also expanded, consistent with the expanded anterior *Krox-20* stripe (r3). Interestingly, the r5 stripe of *Krox-20* becomes thicker in AGN treated embryos (compare with st. 13 in Fig. 2L,M). The *XlPOU2* stripe associated with r4 is no longer distinguishable, as it fuses with the spinal cord domain, from which it is separated by a small gap in control embryos (Fig. 2G).

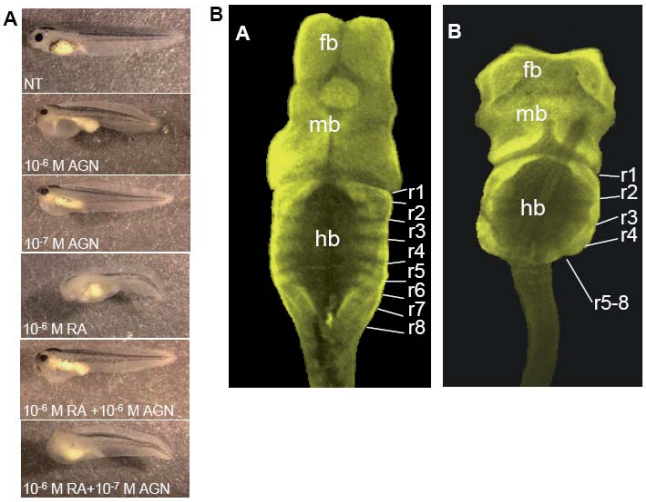


Fig. 1. (A) The RAR antagonist AGN rescues the RA phenotype.
Xenopus laevis embryos incubated in 10^{-6} M RA show anterior truncations. Embryos incubated in 10^{-6} M and 10^{-7} M AGN show a shorter hindbrain area and a large heart oedema with the phenotype being more severe at 10^{-6} M. When embryos are incubated in equal concentrations (10^{-6} M) of RA and AGN the resulting phenotype is more like the AGN phenotype. When 10^{-6} M RA is combined with 10^{-7} M AGN the phenotype is more like an RA phenotype. NT (control). (B) AGN treatment caused severe brain malformations. CLSM images of stage 45 tadpole brains labelled with Xen1 and 2G9 antibodies. (A) Control embryo, treated with 0.1% DMSO. (B) 10^{-6} M AGN treated embryos (**fb**: forebrain; **mb**: midbrain; **hb**: hindbrain, rn refer to rhombomere numbers). In AGN treated embryos, the number of rhombomeres was reduced to 4 or 5.

Co-staining for *Krox-20* and *Hoxc-6* confirms that the posterior hindbrain region is severely shortened after AGN treatment (Fig. 2E). The correlation between these expression pattern changes and the malformations observed in the future hindbrain of AGN-treated embryos suggests that the action of retinoid signalling on gross A-P patterning of the hindbrain is complete by st. 20.

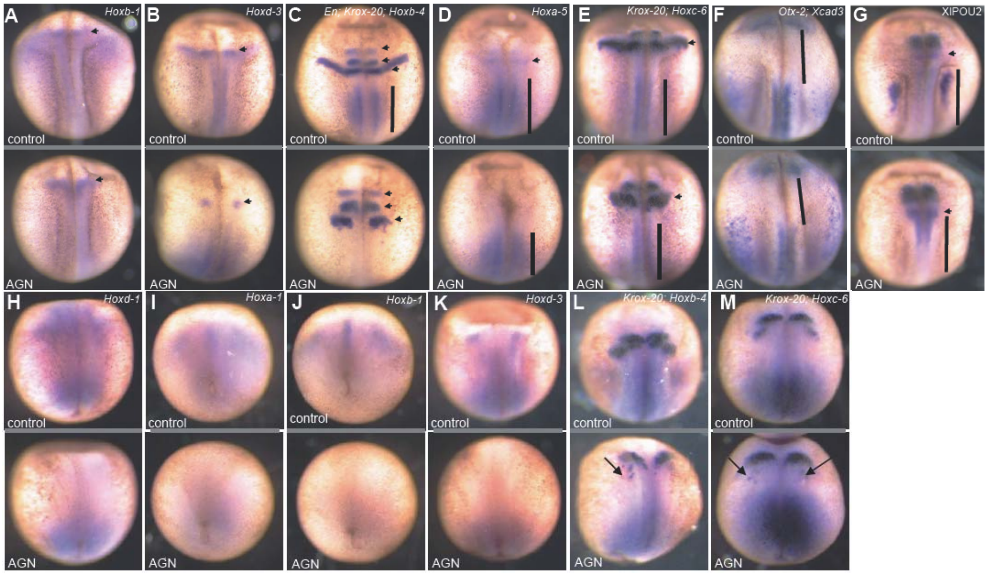
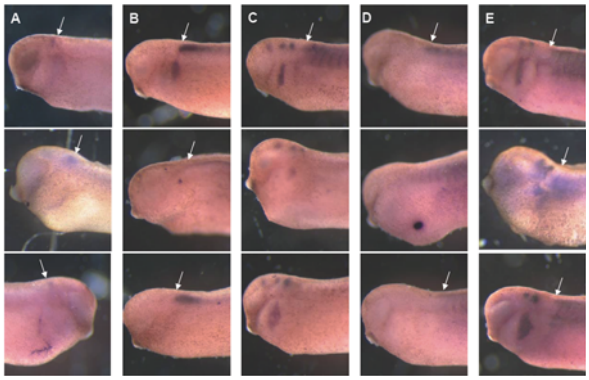


Fig. 2. Top panel shows whole-mount in situ hybridizations (wISH) on st. 20 *Xenopus laevis* embryos (A-F). The upper row shows non-treated embryos (indicated by control) and the bottom embryo is treated with 10^{-6} M AGN (indicated by AGN). All views are dorsal and anterior at the top. (A) *Hoxb-1*, arrowhead indicates hindbrain expression; (B) *Hoxd-3*, arrowhead indicates hindbrain expression; (C) *En2*, *Krox-20* and *Hoxb-4*, top arrowhead indicates *En* stripe, bottom arrowheads indicate *Krox-20* stripes and bar indicates *Hoxb-4* stripe; (D) *Hoxa-5*, arrowhead indicates hindbrain expression and bar indicates spinal cord expression; (E) *Krox-20* and *Hoxc-6*, arrowhead indicates posterior *Krox-20* stripe, bar indicates *Hoxc-6* expression; (F) *Otx-2* and *Xcad3*, bar indicates gap between *Otx-2* (anterior) and *Xcad3* (posterior) expression patterns; (G) *XlPOU2*, arrowhead indicates hindbrain expression and bar indicates spinal cord expression.
Bottom panel shows whole-mount in situ hybridizations on st. 13 *Xenopus laevis* embryos (H-M). The upper row shows non-treated embryos (indicated by control) and the right embryo is treated with 10^{-6} M AGN (indicated by AGN). All views are dorsal and anterior at the top. (H) *Hoxd-1*; (I) *Hoxa-1*; (J) *Hoxb-1*; (K) *Hoxd-3*; (L) *Krox-20* (anterior stripes) and *Hoxb-4*; (M) *Krox-20* (anterior stripes) and *Hoxc-6*. Arrows in pictures L and M localise sparse cells representing the posterior stripe of *Krox-20*.

The hindbrain defect caused by AGN is more extensive in the early neurula

Because considerable evidence indicates that retinoids affect axial patterning during gastrulation and the coincidental expression of genes responsible for establishing a retinoid signalling domain, we looked for the possible effects of abrogation of RA signalling during earlier stages of development. We chose to focus on a time at which anterior (3') *Hox* genes (paralogues 1-6) would normally be expressed and therefore could be affected by an



impaired RA signal. At the early neurula (st. 13) stage, the most 3' of the *Hox* genes examined (*Hoxd-1*, *Hoxa-1*, *Hoxb*-1, *Hoxd-3* and *Hoxb-4*) are strongly affected by reduced retinoid signalling (Figs. 2H-L). However, *Hoxc-6* is unaffected (Fig. 2M). Dissection of embryos confirmed that expression of the most 3' *Hox* genes is severely downregulated in the neuroectoderm upon AGN treatment (data not shown).

Fig. 3. Whole-mount in situ hybridizations on tadpole (st. 32) *Xenopus laevis* embryos. The upper row shows non-treated embryos; the middle row embryos treated with 10^{-6} M AGN from the blastula until the point of fixation; the lowest row embryos treated with 10^{-6} M AGN from st. 13 until the point of fixation. All views are lateral. (A) *Hoxb-1*; (B) *Hoxd-3*; (C) *En2, Krox-20 and Hoxb-4*; (D) *Hoxa-5*; (E) *Krox-20* and *Hoxc-6*. Arrows point to the anterior expression border of each *Hox* gene.

To compare the length of the presumptive posterior hindbrain with and without AGN treatment, a combination of probes was used for in situ hybridisation. After AGN treatment, the gap between the *Krox-20* stripes and *Hoxc-6* is much smaller (Fig. 2M) and the *Krox-20* stripe at r5 is also greatly reduced. This shows that truncation of the posterior hindbrain domain by retinoid depletion is effective already at the end of gastrulation.

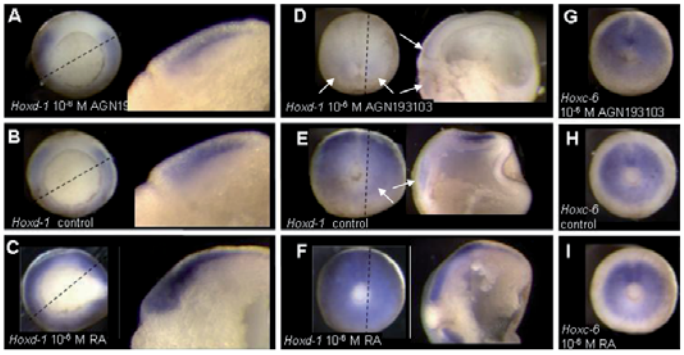


Fig. 4. Whole-mount in situ hybridizations on *Xenopus laevis* embryos. *Hoxd-1* expression at st. 11 (A-C) or st. 12.5 (D-F). Embryos were incubated with 10^{-6} M AGN (A, D), 10^{-6} M RA (C, F) or not treated (0.1% DMSO) (B, E). After photographing the embryos were cut along the indicated dashed line and a lateral view of the cut surface is shown next to the right of each embryo. Arrows in 3D and 3E point to the faint mesodermal expression remaining at that stage (mostly non-involuted mesoderm). *Hoxc-6* expression on stage 12 embryos (G-I). Treatment with 10^{-6} M AGN (G), 10^{-6} M RA (I) or not treated (H).

To correlate the late hindbrain phenotype in the tadpole with the molecular truncation observed in the young neurula, we treated embryos beginning either before or after gastrulation and incubated them until the point of fixation (st. 45). AGN treatment before gastrulation causes the phenotype shown in Figure 1B. Treatment after the end of gastrulation (st. 13 onwards) however, does not cause gross morphological malformations (results not shown). Surprisingly, when we compared the molecular profiles yielded by the

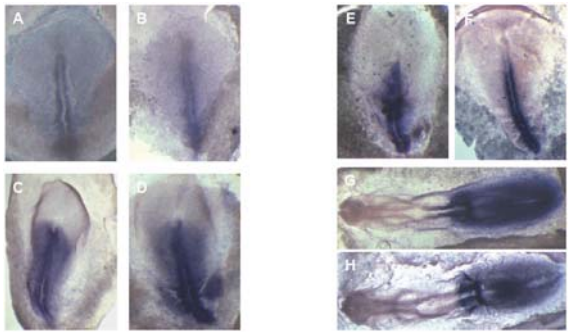


Fig. 5. Whole-mount in situ hybridizations on quail embryos. *Hoxb-1* and *Hoxb-4* expression patterns in quail embryos. Wild-type HH st. 4 early (A) and late (C) are compared to equivalent VAD (B) and (D) embryos; both show expression of *Hoxb-1* in the primive streak and later in migrating ingressed cells. *Hoxb-4* expression patterns are also shown for HH st. 4 wild-type (E) and VAD (F) embryos, along the primitive streak. At HH st. 8 *Hoxb-4* expression pattern includes the neural tube in wild-type (G) but not in VAD (H) embryos. All views are dorsal.

different approaches, we observed that postgastrulation treatment with AGN still causes an alteration, albeit minor, in the molecular pattern of the posterior hindbrain of tadpole embryos. Namely, expression of the most anterior *Hox* genes in the posterior hindbrain (Hoxb1 and Hoxd3) is not affected by retinoid depletion after the end of gastrulation, but the most posterior ones (Hoxb4 and to a lesser extent Hoxb5) are still sensitive to AGN treatment after this period (Fig. 3).

The extension of the posterior hindbrain -indicated by the distance between the posterior expression stripe of krox-20 in r5 and the anterior expression boundary of Hoxc-6 in the spinal cord (Fig. 3E)- is not changed by AGN treatment after gastrulation, as compared to non-treated embryos; on the contrary, AGN treatment beginning before gastrulation causes a remarkable shrinkage of the region, as was already seen at earlier stages. These results indicate that retinoid signalling is required mainly before the end of gastrulation for the hindbrain to acquire a proper morphology; however, part of its pattern remains flexible and it is retinoid dependent..

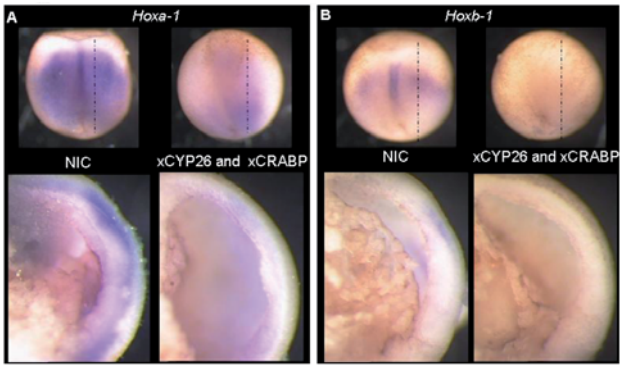


Fig. 6. Whole-mount in situ hybridizations on *Xenopus laevis* embryos. *Hoxa-1* (A) and *Hoxb-1* (B) expression at st. 13. NIC: non-injected controls. xCYP26 and xCRABP: Injection of 100 pg xCYP26 : 100 pg xCRABP mRNA four times, one time into each macromere at 8-cells stage. Whole embryos (top picture) are shown in a dorsal view with anterior being up. Cut embryos (bottom picture) are shown in a lateral view.

Mesodermal* Hox *gene expression during gastrulation is not retinoid dependent but expression of 3' Hox genes in gastrula neurectoderm is

The above experiments suggest that the phenotype observed after AGN treatment in the hindbrain arises during gastrulation, and that retinoid mediated patterning of the

presumptive hindbrain begins at this time. We therefore undertook a more detailed study of the phenotype generated by AGN during gastrulation by analysing both the initiation and the maintenance of *Hox* gene expression during this developmental period.
Hoxd-1 appears as a "pioneer" gene, its expression first becoming detectable at stage 10^{+} (Wacker et al., 2004). AGN treatment does not affect the initial expression in non-organiser mesoderm (Fig. 4A,B). However, as gastrulation proceeds in normal embryos, *Hoxd-1* expression becomes localised more dorsally and is then transferred onto the neuroectoderm; this pattern is absent in AGN treated embryos. By the end of gastrulation, the expression pattern of *Hoxd-1* is very strongly reduced and remains exclusively in its mesodermal domain upon retinoid inhibition (Fig. 4D,E). In contrast, when RA instead of AGN is added to the medium, *Hoxd-1* expression is induced in a much larger domain and earlier than in control embryos; expression is particularly strong in the ectoderm and is maintained throughout gastrulation (Fig. 4C,F).
We then extended the study to *Hoxa-1* and *Hoxb-4*. Both are first expressed weakly at about stage 11 and are upregulated as gastrulation advances (Wacker et al., 2004). We analysed the timing of the consequences of AGN treatment on their expression: whereas their initiation in the mesoderm is not affected, embryos fail to develop normal neuroectodermal expression when incubated in AGN throughout gastrulation (data not shown). Unlike the three genes mentioned above, neither *Hoxc-6* (Fig. 4G,H,I) nor *Hoxb-9* (not shown) expression is affected by either gain- or loss of retinoid function at this stage of development.

Avian embryos also employ a mechanism other than RA to regulate early mesodermal* Hox *expression
The above experiments show that abrogation of the retinoid pathway with a synthetic inhibitor in *Xenopus* embryos impairs the normal neuroectodermal expression of 3' *Hox* genes in the mid-axial region early during development. However, the earliest appearance of the same 3' *Hox* transcripts in mesodermal tissue is not affected by this treatment. To investigate whether such a difference in regulation between the two germ layers is due to limitations of our experimental approach, we turned to the VAD quail model. We performed in situ hybridisation with two 3' *Hox* genes on both VAD and normal quail gastrula and neurula embryos to see the effects of an absolute depletion of retinoid signal (Fig.5). Our results show that expression of *Hoxb-1* and *Hoxb-4* is initiated during gastrulation first in the primitive streak and later in mesodermal cells and that this activation is seen in both control and VAD embryos. In the CNS, expression of these genes begins only after the start of neurulation (Fig. 5G) and is affected in VAD embryos (Fig. 5H). Thus, the avian embryo provides further evidence that 3' *Hox* genes are regulated differently in the mesoderm and in neural tissue. Furthermore, there is an asynchrony of *Hox* expression between the two tissues, which is much more pronounced in avian than in *Xenopus* embryos.
Retinoid dependent transfer of information from mesoderm to neuroectoderm in the gastrula
The above experiments suggest that retinoid signalling may be involved in the transfer of positional information from mesoderm to neuroectoderm in the hindbrain region at early stages of development. To test this more directly we first injected mRNA encoding xCYP26, which is involved in the degradation of RA (Hollemann et al., 1998) into *Xenopus*

early embryos. xCYP26 mRNA alone or, more effectively, co-injection of xCYP26 and xCRABP mRNAs into *Xenopus* restored the axial defects caused by RA incubation. We conclude that combined ectopic expression of *xCYP26* and *xCRABP* causes retinoid loss of function. To test the idea that retinoid signalling from the mesoderm is involved in hindbrain patterning, we loaded gastrula mesoderm cells but not neuroectoderm cells by injection of *xCYP26* and *xCRABP* into all four vegetal blastomeres (fated to become mesoderm and endoderm, but not neuroectoderm; see Figure 8 in Supplementary data) in 8-cell-stage embryos. This causes dramatic loss of *Hoxa-1* and *Hoxb-1* expression in the neuroectoderm (Fig..6). Together, these results strongly suggest that a retinoid-dependent signal from mesoderm is required for neuroectodermal *Hox* expression.

Discussion

The retinoid antagonist AGN193109 gives a strong phenotype in the *Xenopus* CNS, resembling those previously reported using other highly effective approaches to inhibit retinoid signalling (Maden et al., 1996, Kolm et al., 1997; Blumberg et al., 1997; van der Wees et al., 1998, Niederreither et al., 1999; Niederreither et al., 2000, Begemann at al., 2001; Grandel et al., 2002, Hollemann et al., 1998; Kudoh et al., 2002, Dupe and Lumsden, 2001; Hernandez et al., 2007). This phenotype features disturbances of the posterior hindbrain and anterior spinal cord, and is detectable in its definitive form in late neurula stage embryos. This "mid-axial" phenotype arises during gastrulation. AGN treatment during the gastrula stage already causes disturbances of the gastrula stage neural expression of all 3' anterior *Hox* genes examined (*Hoxd-1*, *Hoxa-1*, *Hoxb-1*, *Hoxd-3* and *Hoxb-4*), whereas expression of more 5' posterior *Hox* genes (*Hoxc-6* and *Hoxb-9*) is not affected. Severe changes in gene expression are seen later in the future posterior hindbrain region at the early neurula stage. This early defect is the converse of that caused by early retinoid application (Conlon and Rossant, 1992; Godsave et al., 1998b). The defect is more extensive than that observed at later stages, both in this investigation and in previous retinoid loss of function studies. If this difference is due to a recovery occurring at later stages, this must be independent of retinoid signalling, as retinoid inhibitor treatments from the end of gastrulation to larval stages could not prevent it. Auto- and cross-regulation among *Hox* genes is likely to be involved, reflecting a second *Hox* phase of axial patterning in the CNS. It has been demonstrated in both chicken and mouse that there is indeed a second phase of regulation for *Hox* genes, which starts soon after initiation of somitogenesis and is not dependent on retinoic acid but rather on auto-regulation of and interactions between *Hox* genes (Gould et al., 1998).

In our experiments, virtually no trace of 3' *Hox* mRNA expression (paralogues 1-5) was detectable in the prospective hindbrain region of retinoid antagonist-treated early neurulae (the region that is to recover part of its pattern in the following stages). One possible explanation is that Hox proteins remain in an area and time where the corresponding mRNAs have disappeared; these Hox proteins would be insufficient to activate early neural *Hox* gene expression by means of the known cis-acting HOX responsive elements, since early expression requires retinoid activation via RAREs (see above). However, once retinoid sensitivity ends and RA-independent auto-regulatory and cross-regulatory elements take over neural regulation of 3' *Hox* genes, Hox proteins remaining in the region could still be available in sufficient amounts to trigger the new phase of *Hox* induction. Another

possible explanation is that factors other than Hox proteins themselves are needed to start the second phase of *Hox* activation in the hindbrain region, for instance FGF (Godsave and Durston, 1997) or WNT (McGrew et al., 1997). If a second activation phase devoid of Hox protein remnants in the region occurs, the ordered array of *Hox* expression observed after partial restoration of the initial mid-axial defects would be consistent with *Hox* collinearity. Whatever the explanation, it appears that during early development the embryo retains a safety mechanism, employing a second round of ordered *Hox* activation to allow possible environmental deficits of vitamin A to be circumvented.

We followed the dynamics of the consequences of retinoid antagonist treatment on *Hox* expression during gastrulation. This is precisely the period in which the first transcripts are detected in all vertebrates studied (Deschamps and Wijgerde, 1993; Gaunt and Strachan, 1996); Wacker et al., 2004). Moreover, in *Xenopus*, expression is initiated in non-organiser mesoderm, before appearing in the overlying neuroectoderm. We found that the antagonist compromises neuroectodermal *Hox* expression, but has no effect on non-organiser mesodermal expression. To confirm our observations and at the same time test the universality of this phenomenon in vertebrates, we took advantage of the VAD quail model. These embryos lack endogenous retinoid signalling and therefore offer an alternative approach to antagonist-treatment in *Xenopus laevis*. Analysis by whole-mount in situ hybridisation indicated that early mesodermal expression of two 3' *Hox* genes (*Hoxb-1* and *Hoxb-4*) is not affected in VAD embryos, whereas later neural plate expression is impaired; this phenotype is comparable to that observed in *Xenopus* neurulae. Therefore, our evidence from both *Xenopus* and avian embryos indicates that *Hox* regulation in the early paraxial mesoderm is distinct from that in the prospective hindbrain and independent of retinoid signalling. The nature of this regulation is yet to be elucidated.

We hypothesised that a possible early function for retinoid signalling would be to mediate transfer of A-P information (and thus of *Hox* expression) from mesoderm to neuroectoderm. This idea is supported by many other data, including mesodermal location of the RA generating enzyme RALDH2 in different vertebrates (Swindell et al., 1999; Berggren et al., 1999; Haselbeck et al., 1999; Chen et al., 2001; Begemann et al., 2001) together with neural action of retinoid dependent enhancers (Gould et al., 1998). In addition to that, somite derived retinoid signalling promotes neuronal differentiation in chick embryos (Diez del Corral et al., 2003). We tested this idea by knocking-out mesodermal retinoid signalling but not neural retinoid signalling by targeting mesoderm precursor blastomeres with mixed mRNAs for xCYP26 and xCRABP, two proteins which mediate retinoid degradation. This treatment effectively eliminates the early neural expression of two 3' *Hox* genes examined (*Hoxa-1* and *Hoxb-1*), indicating that retinoid signalling is required for a "vertical" signal (corresponding to part of Nieuwkoop's "transformation" signal) generated by mesoderm and which induces 3' *Hox* genes in neuroectoderm.

The present experiments in *Xenopus* indicate that the retinoid-mediated component of the "transformation" signal acts before the end of gastrulation and arises from non-organiser mesoderm. However, experiments in avian embryos have suggested that the later somitic mesoderm can also signal to impart pattern onto the hindbrain in a retinoid-dependent way (Itasaki et al., 1997; Gould et al., 1998). Moreover, regionalisation of the avian posterior hindbrain seems to coincide with the beginning of somitogenesis (Nordström et al., 2006). This timing difference could be due to distinctive characteristics of each species: in *Xenopus* the mesoderm migrates as a sheet (involution) which may allow for an early and

robust source of signal to pattern the overlying neuroectoderm during gastrulation, whereas the cell-by-cell mode of mesoderm migration in the avian embryo is less favourable for local delivery of the morphogen (RA). We suggest that in *Xenopus* retinoids emanate from the non-segmented non-organiser mesoderm to pattern the overlying neuroectoderm along with gastrulation movements, whereas avian embryos undergo this process later, after re-epithelialisation of the mesoderm into somites can provide a robust retinoid signal to the adjacent neural tissue. This signal might directly mediate a positionally specific vertical instruction or it might have an auxiliary function (Gould et al., 1998). Whatever the mechanism, the mode of action (mesoderm to neuroectoderm) and nature (RA) of the signal appears to be common to both species and it correlates well with the predicted properties of part of the "transformation" signal that Nieuwkoop proposed long time ago for amphibians (Nieuwkoop, 1952).

Materials and methods

Embryo preparation, culture and treatment

Xenopus embryos were obtained by in vitro fertilisation using standard procedures, cultured in 1% MMR containing gentamycin (0.5 g/l) or 0.1% MBS (Sive et al., 1998) and staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1956).

RA and AGN193109 treatment was from stage 8 to stage 13 by culturing embryos in 1% MMR or 0.1% MBS containing RA or AGN193109. Stock solutions were prepared by dissolving RA (Sigma) and AGN193109 (Allergan) in DMSO to concentrations of respectively 10^{-2} M and 10^{-3} M and were stored at -80^{0}C. Final dilutions for embryo incubations were made in 1% MMR or 0.1%MBS.

Xenopus embryos used in whole mount in situ hybridisation analysis were fixed in MEMPFA (Harland, 1991) for 4 h at room temperature or overnight at 4^{0}C, washed once in methanol and stored at -20^{0}C in fresh methanol. Fertilized vitamin A-deficient (VAD) and normal quail embryos were obtained and staged as previously described (Dersch and Zile, 1993; Zile et al., 2000).

Microinjection

For microinjection, one-cell-stage embryos were transferred to 4% ficoll in 1% MMR or 2% ficoll in 0.1% MBS. After injection the embryos were cultured in 4% ficoll, 1% MMR until stage 8/9 and subsequently transferred to 1% MMR with or without ligands to culture the embryos until the appropriate stage.

Lineage tracing

Sulforhodamine dextran (MW 10,000) (S-359, Molecular Probes) was injected in all four macromeres of 8 cells stage embryos, 1 nl of 5 ng/nl each injection, as described above. Embryos were cultured and allowed to develop until st. 40, when they were collected and fixed in MEMPFA, all as described above. They were stored in methanol at -20^{0}C until required. For histology sections, embryos were briefly transferred to 100% ethanol, subsequently cleared in Histo-Clear (National diagnostics) for about 25 min at room temperature, followed by graded immersion in paraffin at 60^{0}C and incubation in 100% paraffin overnight at 60^{0}C. Next day samples were embedded and allowed to solidify at 4^{0}C for approximately 1 hour. Prior to sectioning, they were placed outside the fridge to accommodate to room temperature. Histological sections were performed at 8µm of thickness, placed on slides pre-coated with BioBond according to the manufacturer (Electron Microscopy Sciences), on a drop of distilled water, then mildly heated on a heating plate until sections were nicely stretched; finally, they

were placed at 37^0C to dry completely. Sections were deparaffinised with Histo-Clear, gradually immersed into ethanol and subsequently hydrated, to be mounted in gelvatol containing DAPCO. Analysis and photography was performed by means of an AxioPlan 2 Imaging compound microscope and the corresponding software (Zeiss), provided with a TRITC band-pass filter.

Immunofluorescence and confocal microscopy

For immunostaining of the CNS, embryos were fixed overnight at 4^0C in methanol. Pigmentation was bleached in 80% methanol, 6% H_2O_2, 15 mM NaOH, for approximately 1 hour. After bleaching, the embryos were washed four times 15 min in PBS containing 0.2% Tween, and blocked for 30 min with PBT (0.2% Tween, 3% bovine serum albumin in PBS). Incubation with the anti-neural antibodies 2G9 (Jones and Woodland, 1989b) and Xen-1 (Ruiz I Altaba, 1992b) at 1:1 2G9 and 1:5 Xen-1 in PBT was overnight at 4^0C. The embryos were washed four times 30 min at room temperature in PBT. Incubation with the secondary antibody conjugated to the Cy-5 far-red fluorophore (Jackson Research Labs, Inc.) was overnight at 4^0C. After washing four times 30 min in PBS containing 0.2% Tween at room temperature, the embryos were fixed in methanol and cleared in 1:2 benzyl alcohol:benzyl benzoate. The Cy-5 signal was analysed with confocal laser scanning microscopy (CSLM). Approximately 25 optical sections were recorded for each embryo, and reconstructed into one image.

Luciferase assay

For measuring the luciferase activity 5-10 embryos were homogenised in 100:1 reporter lysis buffer (Promega) and mixed with 300:1 assay buffer (0.1 M potassium phosphate buffer pH 7.8 (KPi), 1 mM DTT, 3 mM ATP and 15 mM $MgSO_4$). The luciferase reaction was started by addition of 100:1 0.1 M KPi, 1 mM DTT and 0.4 mM luciferine. Light units were measured during 10 seconds in a luminometer (Biocounter, Lumac).

In situ hybridisation

Whole-mount in situ hybridisation (w-ISH) on *Xenopus* embryos was performed as previously described (Harland, 1991), except that probe concentration was reduced to 40 ng/ml, hybridisation temperature raised to 65°C and antibody incubations done in 0.1 M Maleic acid, 0.15 M NaCl, 0.1% Tween-20, 1% blocking reagent (Roche), pH 7.5 with anti-Digoxigenin-AP, Fab fragments (Roche). Analysis of the staining pattern was performed in PBS or 1:2 benzyl alcohol:benzyl benzoate to clear the embryos.

w-ISH on quail embryos was performed as previously described (Stern, 1998) for chick embryos.

Templates for RNA synthesis

Xenopus antisense DIG-labelled transcripts were prepared from the following templates: a 1312 bp *Hoxa-1* fragment (*Hoxa-1*), a 666 bp *Hoxb-1* fragment (*Hoxb-1*), xHoxlab1 (*Hoxd-1*) (Sive and Cheng, 1991), EST: dac02e11 (*Hoxd*-3), a 708 bp fragment containing the complete *Hoxb-4* ORF (*Hoxb-4*), EST: XL045g13 (*Hoxa*-5), a 998 bp *Hoxc-6* fragment in pGEM1 containing a part of the homeodomain and extending into the 3' UTR (*Hoxc-6*), a 470 bp *Hoxb-9* fragment in pGEM3 (*Hoxb-9*), a 1400 bp *Krox-20* fragment (*Krox-20*) (Bradley et al., 1993), a 1500bp *Engrailed-2* cDNA (*En-2*) (Hemmati-Brivanlou et al., 1991), the XLPOU 2 ORF (*Xlpou 2*) (Witta et al., 1995), the Xcad-3 ORF (*Xcad-3*) (Pownall et al., 1996), the xCRABP ORF (*xCRABP*) (Dekker et al., 1994) and a 220 bp *OTX-2* fragment *(xOTX-2*) (Pannese et al., 1995).

Chick antisense DIG-labelled transcripts were used for w-ISH on quail embryos, prepared from the following templates: a 2 Kb Hoxb1 cDNA and a Hoxb4.

Acknowledgements

We would like to thank Nobue Itasaki for kindly providing us with the chick Hoxb1 cDNA, as well as Robb Krumlauf for the chick Hoxb4. We are also grateful with Gerda Lamers for her technical assistance and advice on histological sections and microscopy. AJD and CS acknowledge financial support from the EU network of excellence LSHM-CT-2003-504468: Cells into Organs.

MHZ was supported by NIH Grant 5R01 HL61982-03, the National Research Initiative USDA Grant 2005-35200-15257 and the Michigan Agricultural Experiment Station.

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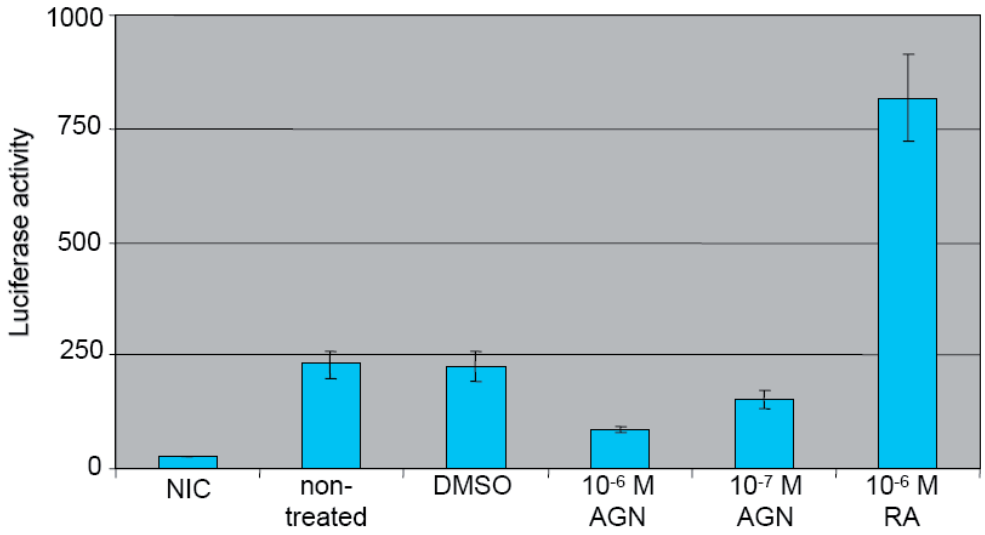
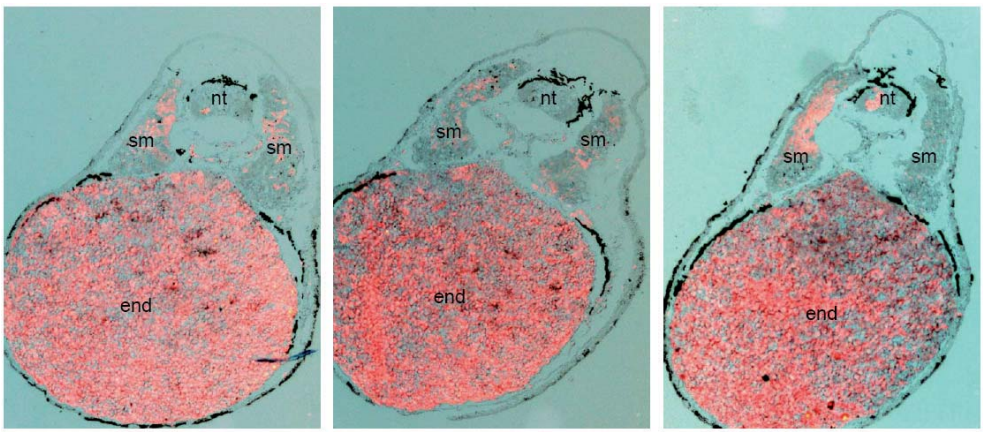


Supplementary Fig. 7. The endogenous retinoid activity in *Xenopus laevis* embryos is reduced by the RAR antagonist AGN193109. Embryos were injected with a DR-5-luciferase reporter construct and cultured in 1% MMR containing 0.1% DMSO (solvent control), 10^{-6} M AGN or 10^{-6} M RA (positive control) from stage 9 to 13. Luciferase activity was analysed by measuring 8 pools of 5 stage 13 embryos. Values shown are average values, represented as relative luciferase activity (non-injected control (NIC) is set at 1). Error bars represent the s.e.m.



Supplementary Fig. 8. Transversal histological sections along the antero-posterior axis of st. 40 *Xenopus laevis* tadpoles. Rhodamine dextran was injected in the 4 macromeres of 8 cells stage embryos. Images show lineage tracing, where the red colour signal corresponding to the fluorescence emitted by rhodamine molecules has been superposed onto a dark interference contrast image of the same section. Sections of three different embryos show the distribution of the rhodamine dextran, which is mostly confined to the endoderm (end) and the somitic mesoderm (sm), but it only appears in a few cells in the neural tube (nt).