Int. J. Dev. Biol. 44: 463-470 (2000)

Original Article

Retinoid signalling acts during the gastrula stages to promote primary neurogenesis

COLIN SHARPE* and KIM GOLDSTONE[1]

Department of Zoology. University of Cambridge, Downing St., Cambridge CB2 3EJ, England

ABSTRACT Retinoid signalling has been manipulated at different developmental stages to identify a critical period in the gastrula embryo for retinoid-dependent primary neurone formation. The expression of retinoid receptor RARα2 in the posterior neuroectoderm of the gastrula embryo is therefore consistent with a role in primary neurogenesis. In addition we show that the expression of *neurogenin-1* and *XDelta-1,* two genes that contribute to the determination of primary neurone cell-fate in the gastrula embryo, respond to retinoid signalling. These results indicate that retinoid signalling is required for an early step in the process of primary neurogenesis. When retinoid signalling is increased, the number of primary neurones increases, but the phenotype is not the same as the neurogenic phenotype that follows the overexpression of a dominant negative form of *XDelta-1*. Whereas increased retinoid signalling expands the width of primary neurone stripes, dominant negative *XDelta-1* increases the density of primary neurones within the stripes. When retinoid signalling is increased and the primary neurone stripes expand, the expression domain of a floorplate marker contracts. Conversely, when retinoid signalling is inhibited, the expression patterns of floorplate markers widen. These results indicate that retinoid signalling acts at an early stage in primary neural development when the fates of different regions of the neuroectoderm are being determined.

KEY WORDS: *Xenopus, retinoid signalling, primary neurones.*

Introduction

Two populations of neurones can be identified in amphibians and fish (Coghill, 1929; Hartenstein, 1989, 1993; Kimmel and Westerfield, 1991). In *Xenopus*, the embryonic primary neurones are specified as early as the gastrula stages (Lamborghini, 1980) and are active little more than one day after fertilisation (Jacobson and Huang, 1985). They are large, few in number and control the earliest tail-flip movements important for hatching and avoidance behaviours. The primary neurones appear to be an adaptation to an early, free-swimming lifestyle, as directly equivalent cells have not been identified in amniotes. In contrast, the smaller secondary neurones exit the cell cycle at later stages of development and contribute to the extensive larval and adult nervous systems (Hartenstein, 1989).

In *Xenopus*, primary neurone formation is the culmination of a sequence of events that begins with the establishment of a prepattern in the neuroectoderm (reviewed in Sasai, 1998). The prepattern determines the expression of *neurogenin* (*Xngnr-1*), a proneural gene that defines the domains of cells that will give rise to the primary motor, inter and sensory neurones (Ma *et al.*, 1996). Delta-Notch signalling is implicated in the selection of primary neurone precursors from these proneural domains (Chitnis *et al.*, 1996). *X-Myt1* expression consolidates a primary neurone fate in the selected precursors (Bellefroid *et al.*, 1996) and is followed by the expression of *X-NeuroD* (Lee *et al.*, 1995) which promotes differentiation. Ultimately the primary neurones differentiate, express marker genes such as neural-specific type-II beta-tubulin (NST)(Oschwald *et al.*, 1991; Chitnis *et al.*, 1995) and extend axonal processes (Jacobsen and Huang, 1985). The remaining neuroectodermal cells continue to divide until stimulated to differentiate during secondary neurogenesis in the larva or adult (Hartenstein, 1989).

Retinoid signalling plays a role in many aspects of development and differentiation (Kastner *et al.*, 1995; Mangelsdorf *et al.*, 1995;

Abbreviations used in this paper: RAR, retinoic acid receptor; RXR, retinoid X receptor; RA, retinoic acid; NST, neural-specific type-II beta-tubulin; RALDH, retinaldehyde dehydrogenase; Xngnr-1, Xenopus neurogenin related; shh, sonic hedgehog; A-P, anterior-posterior.

***Current address and address for correspondence:** Institute of Biomolecular and Biomedical Science. Division of Genes and Developmental Biology, School of Biological Science, University of Portsmouth, St. Michael's Building, Portsmouth PO1 2DY, England. TEL: 44 23 92 84 20 62. FAX: 44 23 92 84 20 53
e-mail: colin.sharpe@port.ac.uk

[1]**Current address:** Wellcome CRC Institute of Cancer and Developmental Biology, Tennis Court Rd, Cambridge, England.

0214-6282/2000/$20.00

Printed in Spain
www.ehu.es/ijdb

Conlon, 1995; Blumberg *et al.*, 1997; Durston *et al.*, 1999). Supplementing embryos with exogenous retinoic acid (RA) has identified CNS patterning as a potential target for retinoid signalling (Durston *et al.*, 1989; Papalopulu *et al.*, 1991; Ruiz i Altaba and Jessell, 1991). More recently, the elimination of the nuclear receptors that mediate retinoid signalling has confirmed a role for RA in anterior-posterior axis formation and in patterning the hindbrain (Kastner *et al.*, 1995; Conlon, 1995). Removing RA, either by dietary regimes (Maden *et al.*, 1996) or by eliminating the RALDH-2 gene that synthesises RA (Neiderreither *et al.*, 1999), gives similar yet distinct defects in hindbrain organisation. Apart from patterning, retinoid signalling can induce the formation of neurones in cultures of embryonal carcinoma cell lines (reviewed in Maden and Holder, 1992) and in some neural stem cells (Takahashi *et al.*, 1999; Scheffler *et al.*, 1999). Within the neural tube, retinoid signalling induces the proliferation of motor neurone precursors adjacent to the limb, and regulates a cell-fate decision in a subset of these cells (Sockanathan and Jessell, 1998). Retinoid signalling is also required to consign some ventro-lateral neural cells to an inter-neurone fate (Pierani, *et al.*, 1999).

We have previously shown that retinoid signalling is required for primary neurone formation, as the expression of a dominant negative retinoid receptor suppresses their formation (Sharpe and Goldstone, 1997). However, these embryos continue to express the general neural marker sox-2 (Sharpe and Goldstone, 2000) and form a recognisable neural tube, indicating that a lack of retinoid signalling does not prevent neuroectoderm formation and morphogenesis. Conversely, there is a dose dependent increase in primary sensory neurones in response to elevated retinoid signalling (Sharpe and Goldstone, 2000). However, the stage at which retinoid signalling exerts these effects and the mechanism by which increased retinoid signalling increases the number of primary neurones is not yet clear.

Results

Retinoid signalling is required during the gastrula stages for the formation of primary neurones

Retinoid signalling was elevated in embryos by injecting synthetic mRNA encoding xRARα2 (NR1B1, Nuclear Receptor Nomenclature Committee, 1999) and xRXRβ (NR2B2) (*RAR/RXR* mRNA) into one cell of the two-cell embryo, followed by the addition of retinoic acid (RA), at different stages of development. This approach was taken because the addition of physiological concentrations of RA, or the injection of retinoid receptors alone have minimal effects on primary neurone formation. In contrast, the combined treatment to elevate retinoid signalling results in increased primary neurogenesis that is restricted to the normal domains within the neuroectoderm (Sharpe and Goldstone, 2000). Addition of RA at stage 9 (late blastula) causes an increase in Isl-1+ primary sensory neurones on the injected side of the embryo. However, the increase is marginal when RA is added at stage12 (mid-gastrula) and undetectable when RA is added at stage 13 (late gastrula embryo)(Fig. 1A).

In a second approach, retinoid signalling was suppressed with citral, an inhibitor of retinoid synthesis (Fig. 1B)(Schuh *et al.*, 1993; Kikonyogo, *et al.*, 1999; Sharpe and Goldstone, 2000). Adding citral at the end of gastrulation has no effect on the formation of primary neurones, though addition at earlier stages reduces the number of neurones that form (Fig. 1B). These results indicate that retinoid signalling is required during the gastrula stages if it is to influence primary neurone formation.

RARα2 is expressed at the right time and place to mediate retinoid signalling in primary neurogenesis

Previous analyses have shown that the predominant RAR transcripts in *Xenopus* gastrulae encode RARα2 (Sharpe, 1992) and

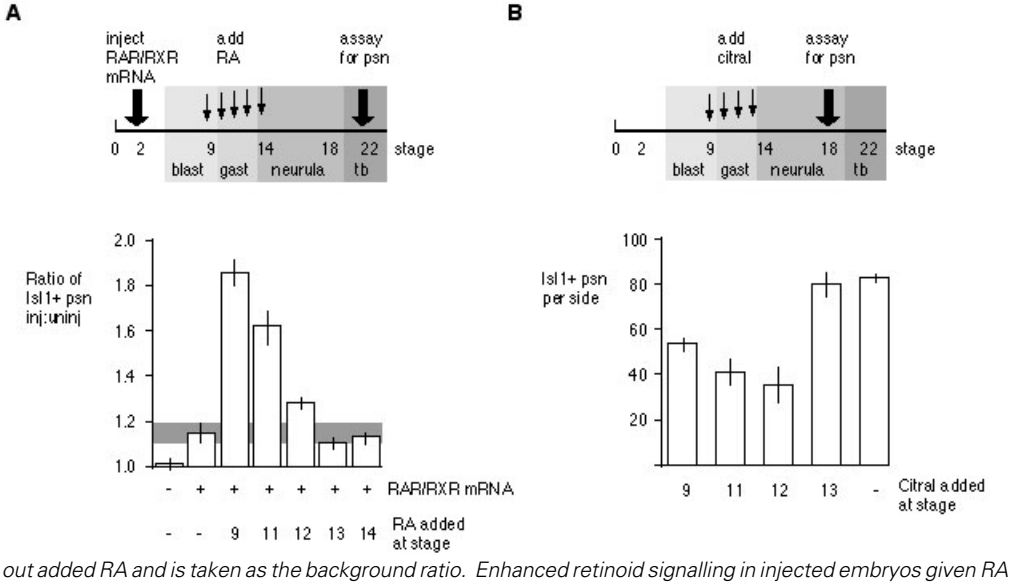

Fig. 1. Retinoid signalling acts during the gastrula stages to regulate primary neurogenesis. *(***A***) Xenopus embryos at the two-cell stage were unilaterally injected with RAR/RXR mRNA and grown to the late blastula stage. 100 nM RA was added to batches of injected embryos at different times across the late-blastula and gastrula stages and the embryos grown to the early tailbud stage. At this point the primary neurones were counted following wholemount staining with an anti-Isl1 antibody. The response to retinoid signalling is expressed as the mean and standard error of the means (SEM) of the ratio of Isl1+ primary sensory neurones on the injected, compared to the uninjected side of the embryo for at least 7 embryos for each treatment. The shaded horizontal bar depicts the SEM for RAR/RXR mRNA injected embryos without added RA and is taken as the background ratio. Enhanced retinoid signalling in injected embryos given RA at stages 9, 11 or 12 resulted in an increase in the number of primary neurones.* **(B)** *Citral, an inhibitor of retinoic acid biosynthesis, was added at a concentration of 60 μM to Xenopus embryos at different time points across the late-blastula and gastrula stages. The embryos were grown to the late neurula stage and assayed for Isl1 expression in the neuroectoderm. Adding citral at stage 13 had no effect on the formation of Isl1+ primary neurones but decreased the numbers of neurones when added at earlier stages. The greater number of neurones at stage 9 compared to stage 10 may indicate the decay of citral before the critical stage of development is reached. In both (***A***) and (***B***) the critical stage for retinoid signalling is during the gastrula stages when primary neurone cell fates are being determined. blast, blastula; gast, gastrula; tb, tailbud.*

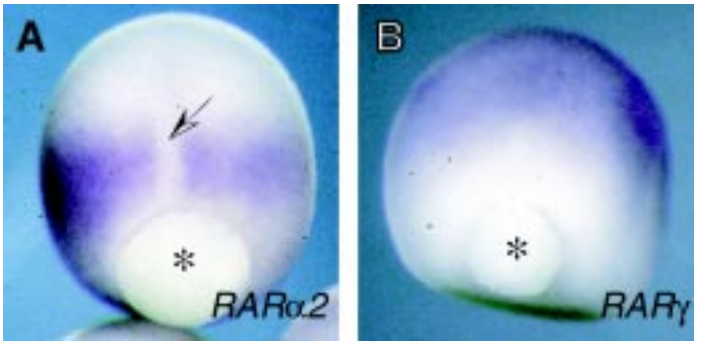

Fig. 2. Localization of RAR transcripts by *in situ* hybridisation. (A) *RARα2 transcripts were found in the posterior neuroectoderm of the mid-gastrula embryo, using a specific antisense probe. Transcripts (purple stain) were found in a single dorsal posterior domain, either side of the future dorsal midline, which itself remains unstained (arrow).* **(B)** *RARγ transcripts were found in neuroectoderm and ectoderm located further from the yolk plug (* in both (**A**) and (**B**)) and crossed the midline.*

RARγ (NR1B3) (Ellinger-Zeigelbauer and Dreyer, 1991). At the mid- to late-gastrula stages when retinoid signalling influences primary neurogenesis, RARα2 transcripts are found in a broad domain that encompasses the posterior neuroectoderm and adjacent epidermis, but not the dorsal midline (Fig. 2A). In contrast, RARγ transcripts are found in a more anterior domain, a region that is not associated with primary neurogenesis (Fig. 2B). Thus RARα2 expression, at both the gastrula stage and in the posterior neuroectoderm, is consistent with a role in an early step of primary neurone formation.

Retinoid signalling influences the expression of genes regulating primary neurogenesis in the gastrula embryo

To determine whether retinoid signalling can influence the expression of genes known to regulate primary neurone formation in the gastrula embryo, we altered retinoid signalling and examined the expression of *neurogenin* (*Xngnr-1*) and *XDelta-1. Xngnr-1* acts as a proneural gene, defining domains within the neuroectoderm that are competent to form primary neurones (Ma *et al.*, 1996) from which the primary neurones are selected in a process mediated by Delta-Notch signalling (Chitnis *et al.*, 1995).

The suppression of retinoid signalling by the injection of a synthetic mRNA encoding a dominant negative form of RARα2 (RARdn) (Sharpe and Goldstone, 1997) results in the inhibition of *Xngnr-1* expression in the proneural domains of the late gastrula embryo (Fig. 3 A,B). Injection of RARdn mRNA also inhibits the expression of *XDelta-1* in the stripes corresponding to the primary neurone domains, but not in the stripes in the anterior neuroectoderm (Fig. 3 D,E). Earlier, in the mid-gastrula embryo, the *Xngnr-1* transcripts are found in a broad domain in the posterior neuroectoderm and RARdn mRNA (Fig. 3C) suppresses this expression. The earliest expression

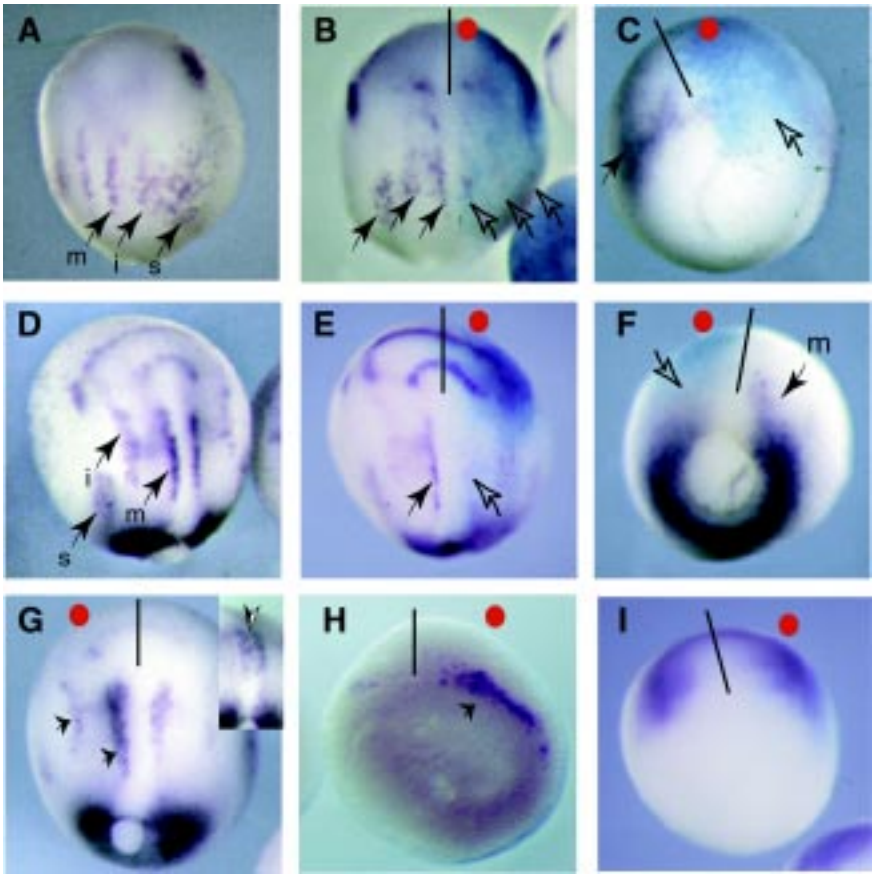

Fig. 3. Retinoid signalling alters the expression of *Xngnr-1* and *XDelta-1,* two genes that are involved in the determination of primary neurone cell-fate. *(**A**) Dorso-lateral view (anterior is to the top in panels (**A**)-(**G**)) of Xngnr-1 expression in a normal late gastrula (stage 13) embryo showing the three domains (arrows) of primary neurogenesis on either side of the midline. m, motor neurone domain; i, inter neurone domain; s, sensory neurone domain. The intense patch of expression at the anterior (top) of the embryo is in the prospective trigeminal ganglion.* **(B)** *Unilateral injection of RARdn mRNA, identified by the activity resulting from co-injected beta galactosidase mRNA (light blue stain, side marked with red dot), suppresses Xngnr-1 expression in each domain of primary neurogenesis (open arrows) compared to the uninjected side (solid arrows).* **(C)** *The early expression of Xngnr-1 at the mid-gastrula stage is also suppressed (open arrow) on the injected side (red dot) compared to expression (solid arrow) on the uninjected side.* **(D)** *In the late gastrula embryo, the expression of XDelta-1 can be resolved into three stripes (arrowed) either side of the midline in the posterior neuroectoderm.* **(E)** *Unilateral injection of RARdn mRNA at the two-cell stage suppresses the expression of XDelta-1 on the injected side (open arrow, red-dot). In contrast, RARdn mRNA does not affect the intensity of XDelta-1 expression in the anterior neuroectoderm.* **(F)** *At the mid-gastrula stage, RARdn activity suppresses the earliest expression of XDelta-1 in the prospective motor neurone stripe (open arrow, red dot). (**G**) Unilateral injection of RAR/RXR mRNA at the two-cell stage expands domains of XDelta-1 expression (small arrows, red dot) in the late gastrula embryo. In some cases the domain of XDelta-1 expression can extend into the dorsal midline region (inset, black and white arrow).* **(H)** *A vegetal view of a mid-gastrula embryo (dorsal to the top and midline marked by line) shows that the unilateral elevation of retinoid signalling also results in the expansion of early Xngnr-1 expression (small arrow). However, injection of RARdn mRNA into one cell of the two-cell embryo does not affect the subsequent expression of Zicr1 (**I**, same orientation as **H**) on the injected side of the embryo (red dot).*

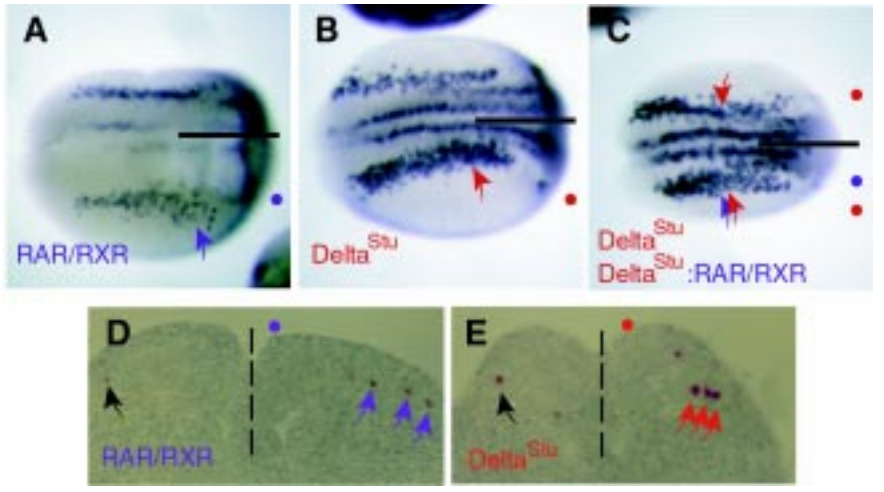

Fig. 4. The extra-neurones phenotype induced by elevated retinoid signalling differs from the *Delta^{stu}* neurogenic mutation. *In* **(A)** *the embryo has been unilaterally injected with RAR/RXR mRNA (purple dot) at the two cell stage and assayed for NST expression at the neurula stage. The striped pattern is maintained but the NST primary sensory neurone domain (purple arrow) is widened in comparison to the uninjected side of the embryo.* **(B)** *In embryos unilaterally injected at the two-cell stage with XDelta^{stu} mRNA (red dot), the primary sensory neurone domain (red arrow) is only slightly wider than the domain in the uninjected half of the embryo, but now the punctate staining is densely packed rather than dispersed as in (A).* **(C)** *Embryos injected into one cell at the two-cell stage with XDelta^{stu} mRNA and into the other cell with a combination of XDelta^{stu} mRNA and RAR/RXR mRNA develop NST expression that is densely packed on both sides, but the stripe on the XDelta^{stu}:RAR/RXR mRNA injected side (red and purple dot) is consistently wider than the NST expression stripe on the side injected with XDelta^{stu} mRNA alone (red dot).* **(D)** *In transverse sections stained for the expression of the primary neurone marker Isl-1, the unilateral injection of RAR/RXR mRNA at the two-cell stage results in an increased number of Isl-1+ primary sensory neurones (purple arrows) on the injected side (purple dot) compared to the uninjected side (dotted line marks the midline). In these embryos the primary neurones on the injected side are well-spaced.* **(E)** *In contrast, whilst the unilateral injection of XDelta^{stu} mRNA also results in the formation of an increased number of primary sensory neurones (red arrows) on the injected side of the embryo (red dot), the neurones are now clustered rather than well-spaced suggesting a failure of lateral inhibition.*

of *XDelta-1* in the neuroectoderm in a stripe corresponding to the prospective primary motor neurones is also suppressed by RARdn mRNA injection, though circumblastoporal expression is unaffected (Fig. 3F). These results suggest that retinoid signalling is required for the expression of *Xngnr-1* and *XDelta-1* in the primary neurone domains during the gastrula stages.

In contrast to the suppression of expression seen following the injection of RARdn mRNA, the elevation of retinoid signalling expands the domains of *XDelta-1* expression (Fig. 3G). In some instances *XDelta-1* expression is seen along the midline of the neuroectoderm (Fig. 3G insert), a region that normally forms floorplate and not primary neurones. Similarly, elevated retinoid signalling expands the domain of expression of *Xngnr-1* in the mid-gastrula embryo (Fig. 3H).

Zicr1 is one of the first genes to be expressed in the neuroectoderm (Mizuseki *et al.*, 1998; Sasai, 1998). In contrast to *Xngnr-1* and *XDelta-1*, the extent of *Zicr1* expression in the gastrula embryo is unaffected by the inhibition of retinoid signalling (Fig. 3I).

The timing experiments and the analysis of gene expression are consistent in their suggestion that retinoid signalling is active, in primary neurogenesis, during the gastrula stages. At this time of development, the decisions that determine which cells will become primary neurones are still being taken and the cells have yet to embark on the process of differentiation (Sasai, 1998).

The extra primary neurones phenotype that forms in response to elevated retinoid signalling is not the same as the neurogenic phenotype generated by loss of lateral inhibition

Mutations in *XDelta-1* that suppress lateral inhibition produce a neurogenic phenotype in which more cells become primary neurones. We have compared the extra-neurones phenotype that accompanies elevated retinoid signalling with the neurogenic phenotype associated with the dominant negative mutant *XDelta^{stu}* (Chitnis *et al.*, 1995).

The extra-neurones phenotype in response to elevated retinoid signalling results in an increase in the width of the bands of NST expression by wholemount *in situ* hybridisation (Fig. 4A). However, within the band the pattern of expression remains punctate. Following *XDelta^{stu}* mRNA injection, the primary neurone stripes are slightly broadened but NST expression is uniform consistent with more cells in the stripe expressing the marker (Fig. 4B). Coinjecting *XDelta^{stu}* and *RAR/RXR* mRNA results in a broadened domain with more uniform NST expression (Fig. 4C), indicating that the two manipulations can act synergistically.

To examine the distribution of neurones within the domains more precisely, we used the anti-Isl-1 monoclonal antibody 39.4D5 (Ericson *et al.*, 1992) that recognises the nuclei of *Xenopus* primary sensory neurones at the late neurula stage (Sharpe and Goldstone, 2000). Unilateral injection of *RAR/RXR* mRNA at the two-cell stage causes a 2-fold increase in Isl-1+ primary sensory neurones and an almost 3-fold increase in the width of the domain in which the neurones are found (Table 1). The neurones, as expected from the NST expression pattern, are dispersed throughout the stripe (Fig. 4D). In contrast, whilst the injection of *XDelta^{stu}* mRNA causes a

TABLE 1

ELEVATED RETINOID SIGNALLING ENLARGES THE PRIMARY NEURONE DOMAIN TO A GREATER EXTENT THAN LOSS OF LATERAL INHIBITION

Comparison	Isl1+ psn/section[2]	fold increase	domain width (μm)[3]	fold increase
RAR/RXR	3.87±1.16	2.04	101.0±10.0	2.7
Normal[1]	1.90±0.95		37.9±16.8	
Delta^{stu}	2.77±1.11	2.05	44.4±11.3	1.9
Normal	1.35±0.78		22.8± 6.7	

(1) Comparisons are between RNA-injected and -uninjected (normal) sides of an embryo. For the RAR/RXR comparison, both sides received 10 nM RA accounting for the increase on the normal sides of these embryos compared to those of the Delta^{stu} injected embryos.
(2) Wax sections cut at 16 μm. Results obtained from n=63 sections derived from three embryos for each comparison.
(3) Data obtained from n≥6 sections for each comparison. Only sections in which the number of Isl1+ primary sensory neurones on both sides was > 1 were used.

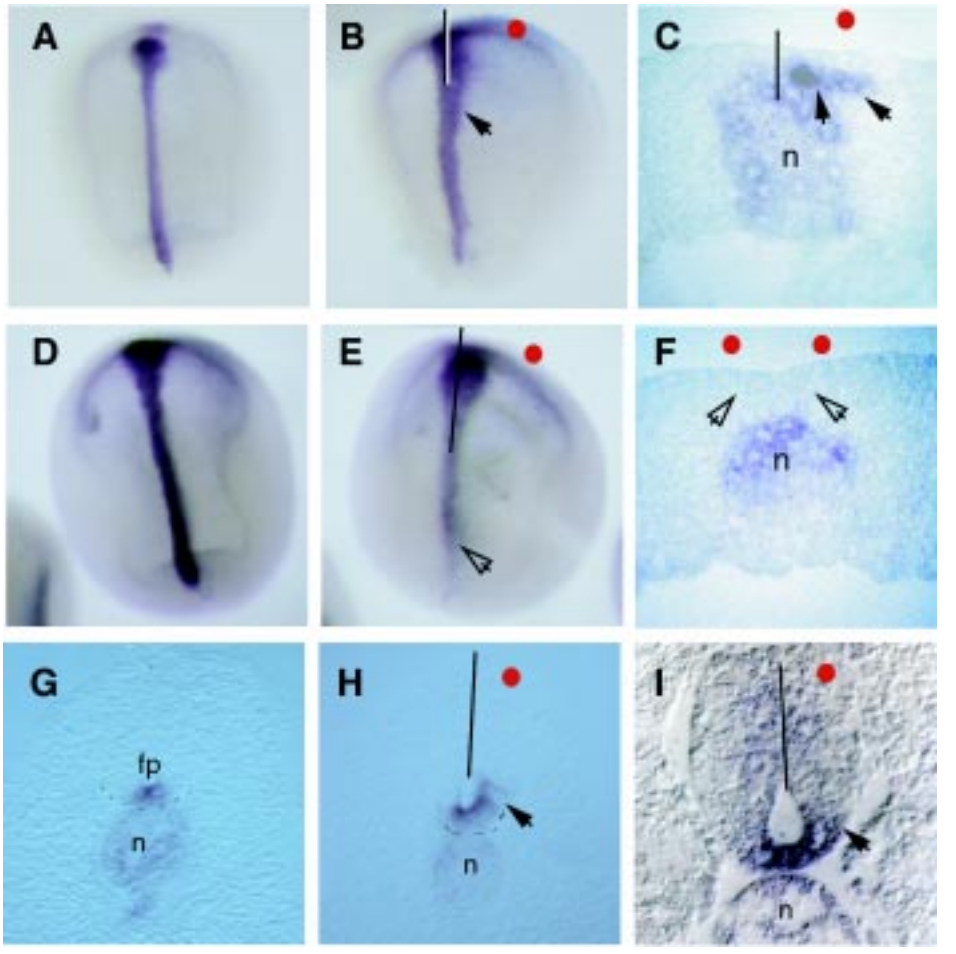

Fig. 5. Retinoid signalling influences the lateral extent of the floorplate. (A) *In situ hybridisation of a neurula stage embryo to show the location of sonic hedgehog (shh) transcripts along the midline. (Anterior to the top in panels A, B and D, E).* **(B)** *Neurula stage embryos unilaterally injected at the two-cell stage with RARdn mRNA show expanded shh expression (arrow) on the injected side (red dot).* **(C)** *Transverse sections through neurula stage embryos show that on the injected side (red dot) shh expression extends from directly over the notochord (n) into adjacent lateral neuroectoderm (arrows). Comparison of* **(D)** *control uninjected and* **(E)** *RAR/RXR mRNA injected neurula stage embryos shows reduced expression (open arrow) of shh on the injected side of the embryo (red dot).* **(F)** *Transverse sections through neurula stage embryos injected into both cells of the two-cell embryo with RAR/RXR mRNA show that shh expression is reduced, and in some cases eliminated, in the neuroectoderm by elevated retinoid signalling. At the tailbud stage, transverse sections through (***G***) control and (***H***) RARdn mRNA injected embryos demonstrate expanded shh expression that extends from the ventral into the ventro-lateral domain of the neural tube (arrowhead). shh expression separated from the ventral floorplate region by non-expressing neural tissue was not seen.* **(I)** *The expression of wnt4 in transverse section of a tailbud embryo treated as in (H) shows expression of wnt 4 extending into the ventro-lateral regions of the neural tube (arrowhead). n, notochord.*

similar 2-fold increase in primary neurons, the width of the domain is increased less than 2-fold (Table 1). In transverse section it is clear that the Isl-1+ primary sensory neurones are densely packed confirming that most, if not all cells, in the domain have become primary neurones (Fig. 4E).

The two procedures therefore appear to generate additional primary neurones through different mechanisms. Elevated retinoid signalling expands the domains but the neurones remain dispersed indicating selection by lateral inhibition. In contrast *XDelta*^{stu} mRNA increases the density of neurones within the domain, suggesting that lateral inhibition is inoperative.

Retinoid signalling influences the expression of shh *in the ventral neural tube*

The injection of RARdn mRNA generates tailbud embryos that lack primary motor neurones and which show a morphological deformation of the ventral neural tube that may represent altered floorplate formation (Sharpe and Goldstone, 1997). We therefore examined whether retinoid signalling can alter the formation of the floorplate by examining the expression of marker genes such as *sonic hedgehog* (Ekker, *et al.*, 1995) and *wnt-4*(McGrew *et al.*, 1992).

Unilateral injection of RARdn mRNA at the two-cell stage results in an enlarged domain of *sonic hedgehog* (*shh*) expression at the neurula stage (Fig. 5 A,B). In transverse sections, *shh* transcripts are detected in a broadened domain that includes regions of the neuroectoderm that do not lie directly over the notochord (Fig. 5C). In contrast, the unilateral injection of *RAR/RXR* mRNA results in a narrower than normal domain of *shh* expression in the neuroectoderm (Fig. 5 D,E), though staining in the underlying notochord remains. The extensive loss of *shh* expression in the neuroectoderm when both cells are injected with *RAR/RXR* at the two-cell stage becomes apparent when the embryos are seen in transverse section (Fig. 5F).

The early injection of RARdn mRNA results, at the tailbud stage, in an expanded domain of *shh* from the most ventral into the ventro-lateral regions of the neural tube (Fig. 5 G,H). In addition, the injection of RARdn mRNA results in an expansion of the *wnt-4* expression (Fig. 5I). The morphology of the neural tube and the expansion of the expression domains of *shh* and *wnt-4* suggest that the level of retinoid signalling may regulate the lateral extent of the floorplate. Consequently, suppressed retinoid signalling can eliminate primary neurone formation and expand the domain allocated to floorplate formation. In contrast increased retinoid signalling increases primary neurone formation but contracts the expression domain of *shh*, a floorplate marker.

Discussion

We have previously shown that retinoid signalling is required for, and can quantitatively regulate, primary neurogenesis in the amphibian neuroectoderm. In this paper we set out to determine

the stage of development at which retinoid signalling influences the formation of primary neurones and investigate the mechanism by which it exerts its effects.

The timing of retinoid signalling during primary neurogenesis

Two techniques have been used to determine the time at which retinoid signalling is required. The first relies on the ability of retinoid signalling to quantitatively activate primary neurogenesis (Sharpe and Goldstone, 2000). The second approach used citral to inhibit retinoid synthesis (Schuh *et al*., 1993; Kikonyogo *et al*., 1999; Sharpe and Goldstone, 2000) and the result using both approaches is strikingly consistent. In each case the critical period for retinoid signalling is during the gastrula stages, when neural cell-fate decisions are taking place, rather than during the neurula stages when primary neurones undergo differentiation. This is similar to the critical period for the acquisition of resistance to retinoid signalling in A-P patterning, which develops over the gastrula and early neurula stages (Sive *et al*., 1990).

The levels of transcripts that encode retinoid receptors are regulated during early development. The maternal transcript for the RARα1 isoform is lost before gastrulation to be replaced by zygotically transcribed RARα2 (Blumberg *et al*., 1992; Sharpe, 1992). We have extended previous observations (Sharpe, 1992; Kolm *et al*., 1997) to show that RARα2 transcripts are found in a posterior domain that includes the caudal neuroectoderm as early as the mid-gastrula stage, with the notable exception of the midline. Unlike RARγ (Ellinger-Zeigelbauer and Dreyer, 1991), which is expressed in a dorsal anterior domain, RARα2 transcripts are, significantly, found both in the right place and at the right time to mediate the retinoid signalling that regulates primary neurogenesis.

If retinoid signalling is required during the gastrula stages, then it should influence the expression of genes that mediate the early steps of primary neurogenesis. We have shown that retinoid signalling alters the expression of two genes, *Xngnr-1* (Ma *et al*., 1996) and *XDelta-1* (Chitnis *et al*., 1995) that are directly involved in primary neurone cell-fate assignation. Both are first expressed in the mid- to late-gastrula stages consistent with our estimate of the time of action of retinoid signalling. Furthermore, we have shown that the earliest expression of both *Xngnr-1* and *XDelta*-1 requires retinoid signalling, suggesting that the initiation of their expression is dependent on retinoid signalling. However, retinoid signalling does not alter the expression of *Zicr1* (Mizuseki *et al* 1998; Sasai, 1998) a gene expressed in the neurectoderm in response to noggin. These results point to a role for RARα2-mediated retinoid signalling at an early stage in primary neurone formation, but downstream of neural induction by noggin.

The extra-neurones phenotype of enhanced retinoid signalling is not the same as the* XDelta^{stu} *neurogenic phenotype

Retinoid signalling expands primary neuronal domains, but retains the dispersed pattern of primary neurone formation. In contrast *XDelta^{stu}* mRNA causes less expansion but results in the dense packing of neurones. This suggests that the response to elevated retinoid signalling is an alteration in the patterning of the neuroectoderm that results in larger domains of primary neurogenesis rather than an alteration in the process of precursor selection from the allocated pool of cells. The simplest explanation is that retinoid signalling is acting upstream of lateral inhibition. This is consistent with recent findings that high levels of exogenous RA cannot rescue the reduced-neurones phenotype associated with the expression of a constitutively active Notch construct (Franco *et al*., 1999).

Does retinoid signalling influence prepattern formation in the neuroectoderm?

The expression of *XDelta-1* (Fig. 3G inset) and NST (data not shown) across the midline of embryos with elevated retinoid signalling suggests that the expansion of the primary neurone domain may be at the expense of other neuroectodermal fates. To examine this possibility we analysed floorplate formation. Elevated retinoid signalling reduced the extent of *shh* expression, a marker of floorplate (Ekker *et al*., 1995), whilst depressed retinoid signalling expanded the expression of both *shh* and *wnt-4* in the ventral neural tube. However, additional *shh* and *wnt-4* expression is not found separated from the ventral-most part of the neural tube. This contrasts with *Pintallavis* mRNA which can induce isolated patches of cells at the dorsal midline to express floorplate markers (Ruiz I Altaba *et al*., 1993). This suggests that retinoid signalling cannot induce floorplate as such, but is involved in the process that determines the extent of the floorplate. The expansion of NST expression and Isl-1staining in the primary sensory neurone stripe suggests elevated retinoid signalling may have a similar role at the edge of the neural plate though it is not yet apparent whether this is at the expense of neural crest or some other neural cell type.

The expression of two genes, *Gli3* and *Zic2* that contribute to the formation of the neuroectodermal prepattern (Brewster *et al*., 1998) is altered in the neurula stage embryo by high levels of exogenous RA (Franco *et al*., 1999). However, the developmental events that lead to a prepattern in the *Xenopus* neurectoderm have not been clearly defined. A range of genes including Zicr1 (Mizuseki *et al*., 1998), the *Xenopus* Iroquois homologues (Mayor *et al*., 1998; Bellefroid *et al*., 1998), the *Xenopus* Hox genes (Kolm *et al*., 1997), early expressed bHLH genes such as HEN-1, the Lim only gene LMO-3 (Bao *et al*., 2000), and Notch (Coffman *et al*., 1993) in addition to Gli3 and Zic2 (Brewster *et al*., 1998) are expressed in the gastrula stage neuroectoderm and may play a role in this process. The consequences of altered retinoid signalling on the expression of these potential prepattern genes at the key gastrula stage is unknown. It is likely that the interaction between retinoid signalling and neuroectodermal prepatterning will require the identification of the direct target genes of retinoid signalling in the gastrula embryo.

In this paper we have shown that retinoid signalling is required during the gastrula stages for the formation of primary neurones. Retinoid signalling appears to act at this stage by influencing the size of the domains committed to primary neurone or, in the ventral neuroectoderm, floorplate fate. The results are consistent with a role for retinoid signalling in the establishment of a prepattern in the gastrula neuroectoderm.

Materials and Methods

Maintenance and treatment of embryos

Xenopus embryos were dejellied in 2% cysteine-HCl (pH 8.0) and grown in 0.1xMBS (Gurdon, 1977). Embryos were selected at required stages according to the Normal Table of Development (Nieuwkoop and Faber, 1994). For the timing experiments, injected embryos were transferred to 0.1x MBS containing 100 nM all-trans retinoic acid (RA)(Sigma) which had been diluted from a 10 mM stock in DMSO. Citral, (3,7-dimethyl-2, 6-octdienal) was prepared in ethanol and diluted 1000 fold in 0.1x MBS to a final concentration of 50 μM. Embryos were grown in these media until the

late neurula stage for neural-specific type-II beta-tubulin (NST) assay, or the early tailbud (stage 22) for Isl-1 wholemount immunocytochemistry.

Injection of synthetic mRNA

Synthetic, capped mRNAs encoding xRARα2, xRXRβ, dominant negative xRARα2 (RARdn) and cytoplasmic beta galactosidase were transcribed using SP6 polymerase (Krieg and Melton, 1988) from coding sequences cloned into the non-coding regions of a *Xenopus* globin cDNA vector (pING14, Bannister *et al.*, 1991; Sharpe and Goldstone, 1997). Synthetic mRNA was prepared at a final concentration of up to 100 ng/nl in water and 10 nl injected into one cell of a two-cell embryo grown in 1xMBS, 3% Ficoll using a Medical Systems Corp. Picoinjector. At the blastula stage, embryos were transferred back to 0.1x MBS. Standard histochemical staining (Turner and Weintraub, 1994) was used to detect co-injected beta-galactosidase mRNA, which acts as a lineage tracer.

Analysis of phenotypes

The expression patterns of NST (Oschwald *et al.*, 1991), xRARα2 (Sharpe, 1992), xRARγ (Ellinger-Zeigelbauer and Dreyer, 1991), XDelta-1 (Chitnis *et al.*, 1995), Xngnr-1 (Ma *et al* 1996), Zicr1 (Mizuseki *et al.*, 1998), shh (Ekker *et al.*, 1995) and wnt-4 (McGrew *et al.*, 1992) were determined by wholemount *in situ* hybridisation (Harland *et al.*, 1991) of MEMFA fixed embryos using the variations suggested by Baker *et al.* (1995). Primary sensory neurones were detected using the monoclonal antibody 39.4D5 (Developmental Studies Hybridoma Bank, University of Iowa) as described in Sharpe and Goldstone (2000). For sectioning, fixed embryos in methanol were transferred to PEDS wax (Sharpe and Goldstone, 1997) and blocks cut at 15-17 microns on a rotary microtome.

Acknowledgements

The anti-Isl1 antibody 39.4D5, developed in the laboratory of Dr T. Jessell was obtained from the Developmental Studies Hybridoma Bank maintained by the University of Iowa, Department of Biological Sciences. We are grateful to Eric Bellefroid, Nancy Papalopulu, Domingos Henrique, Horst Grunz, Stephen Ekker and Lynn McGrew for supplying markers. We thank Mike Taylor and the Basement Developmental Biology Group for helpful discussion, Matt Guille and Melanie Sharpe for comments on the manuscript. An MRC Senior fellowship and an award from the Balfour Fund at the Department of Zoology, University of Cambridge, supported this work.

References

BAKER, C.V.H., TORPEY, N.B., SHARPE, C.R., HEASMAN, J. and WYLIE, C.C. (1995). A *Xenopus* c-kit related receptor tyrosine kinase expressed in migrating stem cells of the lateral line system. *Mech. Dev.* 50: 217-228.

BANNISTER, A.J., COOK, A. and KOUZARIDES, T. (1991). *In vitro* DNA binding activity of Fos/Jun and BZLF1 but not C/EBP is affected by redox changes. *Oncogene* 6: 1243-1250.

BAO, J., TALMAGE, D.A., ROLE, L.W. and GAUTIER, J. (2000). Regulation of neurogenesis by interactions between HEN1 and neuronal LMO proteins. *Development* 127: 425-435.

BELLEFROID, E.J., BOURGUIGNON, C., HOLLEMANN, T., MA, Q., ANDERSON, D.J., KINTNER, C.K. and PIELER, T. (1996). X-MyT1, a *Xenopus* C2HC-Type zinc finger protein with a regulatory function in neuronal differentiation. *Cell* 87: 1191-1202.

BELLEFROID, E.J., KOBBE, A., GRUSS, P., PIELER, T., GURDON, J.B. and PAPALOPULU, N. (1998). Xiro3 encodes a *Xenopus* homolog of the *Drosophila* Iroquois genes and functions in neural specification. *EMBO J.* 17: 191-203.

BLUMBERG, B., BOLADO, J., MORENO, T.A., KINTNER, C., EVANS, R.M. and PAPALOPULU, N. (1997). An essential role for retinoid signaling in anteroposterior neural patterning. *Development* 124: 373-379.

BLUMBERG, B., MANGELSDORF, D.J., DYCK, J.A., BITTNER, D.A., EVANS, R.M. and DEROBERTIS, E.M. (1992). Multiple retinoid-responsive receptors in a single cell: Families of retinoid X receptors and retinoic acid receptors in the *Xenopus* egg. *Proc. Natl. Acad. Sci. USA* 89: 2321-2325.

BREWSTER, R., LEE, J. and RUIZ I ALTABA, A. (1998). Gli/Zic factors pattern the neural plate by defining domains of cell differentiation. *Nature* 393: 579-583.

COFFMAN, C.R., SKOGLUND, P., HARRIS, W.A. and KINTNER, C.R. (1993). Expression of an extracellular deletion of Xotch diverts cell fate in *Xenopus* embryos. *Cell* 73: 659-671.

COGHILL, G.E. (1929). Anatomy and the problem of behaviour. *Cambridge University Press.*

CONLON, R.A. (1995). Retinoic acid and pattern formation in the vertebrates. *Trend. in Genet.* 11: 314-319.

CHITNIS, A., HENRIQUE, D., LEWIS, J., ISH-HOROWICZ, D. and KINTNER, C.R. (1995). Primary neurogenesis in *Xenopus* embryos regulated by a homologue of the *Drosophila* neurogenic gene Delta. *Nature* 375: 761-766.

DURSTON, A.J., TIMMERMANS, J.P.M., HAGE, W.J., HENDRIKS, H.F.J., DEVRIES, N.J., HEIDEVELD, M. and NIEUWKOOP, P.D. (1989). Retinoic acid causes an anteroposterior transformation in the developing central nervous system. *Nature* 340: 140-144.

DURSTON, A.J., VAN DER WEES, J., PIJNAPPEL, W.W. and GODSAVE, S.F. (1999). Retinoids and related signals in early development of the vertebrate central nervous system. *Curr. Top. Dev. Biol.* 40: 111-175.

EKKER, S.C., MCGREW, L.L., LAI, C-J., LEE, J.J., VON KESSLER, D.P., MOON, R.Y. and BEACHY, P.A. (1995). Distinct expression and shared activities of members of the hedgehog family of *Xenopus* laevis. *Development* 121: 2337-2347.

ELLINGER-ZIEGELBAUER, H. and DREYER, C. (1991). A retinoic acid receptor expressed in the early development of *Xenopus* laevis. *Genes Dev* 5: 94-104.

ERICSON, J., THOR, S., EDLUND, T., JESSELL, T.M. and YAMADA, T. (1992). Early stages of motor neuron differentiation revealed by expression of homeobox gene Islet-1. *Science* 256: 1555-1560.

FRANCO, P.G., PAGANELLI, A.R., LOPEZ, S.L. and CARRASCO, A.E. (1999). Functional association of retinoic acid and hedgehog signaling in *Xenopus* primary neurogenesis. *Development* 126: 4257-4265.

GOMEZ-SKARMETA, J.L., GLAVIC, A., DE LA CALLE-MUSTIENES, E., MODOLELL, J. and MAYOR, R. (1998). Xiro, a *Xenopus* homolog of the *Drosophila* Iroquois complex genes, controls development at the neural plate. *EMBO J.* 17: 181-190.

GURDON, J.B. (1977). Methods for nuclear transplantation in Amphibia. *Methods Cell Biol.* 16: 125-139.

HARLAND, R.M. (1991). *In situ* hybridisation: an improved wholemount method for *Xenopus* embryos. *Methods in Enzymol.* 36: 675-685.

HARTENSTEIN, V. (1989). Early neurogenesis in *Xenopus*: The spatio-temporal pattern of proliferation and cell lineages in the embryonic spinal cord. *Neuron* 3: 399-411.

HARTENSTEIN, V. (1993). Early pattern of neuronal differentiation in the embryonic brainstem and spinal cord. *J. Comp. Neurol.* 328: 213-231.

JACOBSON, M. and HUANG, S. (1985). Neurite outgrowth traced by means of horseradish peroxidase inherited from neuronal ancestral cells in frog embryos. *Dev. Biol.* 110: 102-113.

KASTNER, P., MARK, M. and CHAMBON, P. (1995). Nonsteroid Nuclear Receptors: What are genetic studies telling us about their role in real life? *Cell* 83: 859-869.

KIKONYOGO, A., ABRIOLA, D.P., DRYJANSKI, M. and PIETRUSKO, R. (1999). Mechanism of inhibition of aldehyde dehydrogenase by citral, a retinoid antagonist. *Eur. J. Biochemistry* 262: 704-712.

KOLM. P.J., APEKIN, V. and SIVE , H. (1997). *Xenopus* hindbrain patterning requires retinoid signaling. *Developmental Biology* 192: 1-16.

KRIEG, P.A. and MELTON, D.A. (1987). *In vitro* RNA synthesis with SP6 RNA polymerase. *Method. in Enzymol.* 155: 397-415.

LAMBORGHINI, J.E. (1980). Rohan-Beard cells and other large neurons in *Xenopus* embryos originate during gastrulation. *J. Comp. Neurol.* 189: 323-333.

LEE, J.E., HOLLENBERG, S.M., SNIDER, D.L, TURNER, D.L., LIPNICK, N. and WEINTRAUB, H. (1995). Conversion of *Xenopus* ectoderm into neurons by NeuroD, a basic helix-loop-helix protein. *Science* 268: 836-842.

MA, Q., KINTNER, C. and ANDERSON, D.J. (1996). Identification of neurogenin a vertebrate neuronal determination gene. *Cell* 87: 43-52.

MADEN, M. and HOLDER, N. (1992). Retinoic acid and the development of the

nervous system. *Bioessays* 14: 431-438.

MADEN, M., GALE, E., KOSTETSKII, I. and ZILE, M. (1996). Vitamin A-deficient quail embryos have half a hindbrain and other neural defects. *Current Biology* 6: 417-426.

MANGELSDORF, D.J and EVANS R.M. (1995). The RXR heterodimers and orphan receptors. *Cell* 83: 841-850.

MCGREW, L.L., OTTE, A.P. and MOON, R.T. (1992). Analysis of Xwnt-4 in embryos of *Xenopus* laevis: a Wnt family member expressed in the brain and floor plate. *Development* 115: 463-473.

MIZUSEKI, K., KISHI, M., MATSUI, M., NAKANISHI, S. and SASAI, Y. (1998). *Xenopus* Zic-related 1 and Sox-2, two factors induced by chordin, have distinct activities in the initiation of neural induction. *Development* 125: 579-587.

NIEDERREITHER, K., SUBBARAYAN, V., DOLLE,P. and CHAMBON, P. (1999). Embryonic retinoic acid synthesis is essential for early mouse post-implantation development. *Nat. Genet.* 21: 444-448.

NIEUWKOOP, P.D. and FABER, J. (1994). Normal Table of *Xenopus* laevis (Daudin). 2nd edition. North Holland Publishing Co. Amsterdam.

NUCLEAR RECEPTORS NOMENCLATURE COMMITTEE. (1999). A unified nomenclature system for the nuclear receptor superfamily. *Cell* 97: 161-163.

OSCHWALD, R., RICHTER, K. and GRUNZ, H. (1991). Localization of a nervous system-specific class II beta-tubulin gene in *Xenopus* laevis embryos by whole-mount *in situ* hybridization. *Int. J. Dev. Biol.* 35: 399-405.

PAPALOPULU, N., CLARKE, J.D.W., BRADLEY, L., WILKINSON, D., KRUMLAUF, R. and HOLDER, N. (1991). Retinoic acid causes abnormal development and segmental patterning of the anterior hindbrain in *Xenopus* embryos. *Development* 113: 1145-1158.

PIERANI, A., BRENNER-MORTON, S., CHIANG, C. and JESSELL, T.M. (1999). A sonic hedgehog-independent, retinoid activated pathway of neurogenesis in the ventral spinal cord. *Cell* 97: 903-915.

RUIZ I ALTABA, A. and JESSELL, T.M. (1991). Retinoic acid modifies the pattern of cell differentiation in the central nervous system of neurula stage *Xenopus* embryos. *Development* 112: 945-958.

RUIZ I ALTABA, A., COX, C., JESSELL, T.M. and KLAR, A. (1993). Ectopic neural expression of a floor plate marker in frog embryos injected with the midline transcription factor Pintallavis. *Proc. Natl. Acad. Sci. USA* 90: 8268-8272.

SASAI, Y. (1998). Identifying the missing links; genes that connect neural induction and primary neurogenesis in vertebrate embryos. *Neuron* 21: 456-458.

SCHEFFLER, B., HORN, M., BLUMCKE, I., LAYWELL, E.D., COOMES, D., KUKEHOV, V.G. and STEINDLER, D.A. (1999). Marrow-mindedness: a perspective on neuropoiesis. *Trends Neurosci.* 22: 348-357.

SCHUH, T.J., HALL, B.L., KRAFT, J.C., PRIVALSKY, M.L. and KIMELMAN, D. (1993). V-erbA and citral reduce the teratogenic effects of all-trans retinoic acid and retinal respectively in *Xenopus* embryos. *Development* 119: 785-798.

SHARPE, C.R. (1992). Two isoforms of retinoic acid receptor expressed during *Xenopus* development respond to retinoid signalling. *Mech. Development* 39: 81-93.

SHARPE, C.R. and GOLDSTONE, K. (1997). Retinoid receptors promote primary neurogenesis in *Xenopus*. *Development* 124: 515-523.

SHARPE, C.R. and GOLDSTONE, K. (2000). The control of *Xenopus* embryonic primary neurogenesis is mediated by retinoid signalling in the neurectoderm. *Mech. Development* 91: 69-80.

SIVE, H.L., DRAPER, B.W., HARLAND, R.M. and WEINTRAUB, H. (1990). Identification of a retinoic acid-sensitive period during primary axis formation in *Xenopus* laevis. *Genes Dev.* 4: 932-942.

SOCKANATHAN, S. and JESSELL, T.M. (1998). Motor neuron-derived retinoid signaling specifies the subtype identity of spinal motor neurons. *Cell* 94: 503-514.

TAKAHASHI, J., PALMER, T.D. and GAGE, F.H. (1999). Retinoic acid and neurotrophins collaborate to regulate neurogenesis in adult-derived neural stem cell cultures. *J Neurobiol.* 38: 65-81.

TURNER, D.L. and WEINTRAUB, H. (1994). Expression of achaete-scute homolog 3 in *Xenopus* embryos converts ectodermal cells to a neural fate. *Genes Dev.* 8: 1434-1447.

Received: April 2000
Accepted for publication: July 2000