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Retinoid receptors promote primary neurogenesis in *Xenopus*

C. R. Sharpe* and K. Goldstone

Department of Zoology, University of Cambridge, Downing Street, Cambridge, CB2 3EJ, UK

*Author for correspondence (e-mail: crs13@mole.bio.cam.ac.uk)

SUMMARY

Retinoid receptors, which are members of the nuclear hormone receptor superfamily, act as ligand-dependent transcription factors. They mediate the effects of retinoic acid primarily as heterodimers of retinoic acid receptors (RARs) and retinoid X receptors (RXRs). To analyse their function, xRXRβ synthetic mRNA was injected into *Xenopus* embryos in combination with normal and mutated xRARα transcripts. Two informative phenotypes are reported here. Firstly, over-expression of xRXRβ with xRARα results in the formation of ectopic primary neurons. Secondly, blocking retinoid signalling with a mutated xRARα results in a lack of primary neurons. These two phenotypes, from contra-acting manipulations, indicate a role for retinoid signalling during neurogenesis.

Key words: retinoid receptors, neural development, *Xenopus*, transcription factor, neurogenesis

INTRODUCTION

Retinoic acid (RA) is a derivative of vitamin A and has dramatic effects on embryonic development in many species, causing a range of defects, particularly associated with anterior-posterior patterning (reviewed in Linney, 1992; Conlon, 1995). Biochemical analyses have detected retinoic acid and related retinoids in the early embryo (Chen et al., 1994; Creech-Kraft et al., 1994; Pijnappel et al., 1993; Blumberg et al., 1996; Achkar et al., 1996), whilst molecular techniques have identified many of the proteins that mediate retinoid activity. Of these the retinoid receptors act as ligand-dependent transcription factors controlling gene expression in response to RA (reviewed in Mangelsdorf and Evans, 1995). The retinoid receptors are members of the nuclear hormone receptor superfamily and are divided into two distinct groups, the RXRs and the RARs, with three genes (α, β and γ) in each group. Retinoid signalling is likely to be mediated by heterodimers of RXR and RAR, though homodimeric RXRs may also play a role (reviewed in Kastner et al., 1995). Alternative splicing of each gene can generate a range of isoforms, for example, RARα1 and RARα2 transcripts from the RARα gene encode proteins with different amino terminal domains (Leroy et al., 1991).

Retinoid receptor gene expression is regulated during *Xenopus* development. Before gastrulation the embryo contains transcripts encoding RARγ and the RARα1 isoform together with those for RXRα and RXRγ (Blumberg et al., 1992). Following gastrulation, RARγ transcripts continue to be expressed (Ellinger-Zeigelbauer and Dreyer, 1991), but RARα1 is down-regulated and replaced by RARα2 (Sharpe, 1992). Only low levels of RXRα and essentially no RXRγ transcripts persist past the onset of gastrulation (Blumberg et al., 1992), but RXRβ is transcribed throughout early development (Marklew et al., 1994) and is therefore likely to be the predominant RXR isoform during the late gastrula and neurula stages.

The role of retinoid receptors has been examined in mice by the creation of null mutations (Mendelsohn et al., 1994; reviewed in Kastner et al., 1995). These have been engineered to eliminate individual splice isoforms in some instances, or the entire gene in others. In addition, lines of mice have been crossed to produce offspring with null mutations in more than one RAR gene, or lacking both an RAR and an RXR gene. This meticulous approach has generated a vast amount of data demonstrating the importance of retinoid receptors in mediating the wide range of effects that are dependent upon retinoid signalling. The congenital malformations in these mice can be divided into two groups, those showing similarity to the vitamin A deficiency syndrome, and those that do not (Kastner et al., 1995). However, neurogenesis does not seem to be affected, which is surprising given the ability of exogenous RA to perturb neural development (Durston et al., 1989; Papalopulu et al., 1991; Holder and Hill 1991; Marshall et al., 1992).

In *Xenopus* embryos the first or 'primary' neurons differentiate extremely rapidly to provide a rudimentary nervous system (Hartenstein, 1989, 1993). The earliest of these cells to differentiate withdraw from the cell cycle towards the end of gastrulation (Lamborghini, 1980) and begin to send out axons little more than one day later (Jacobson and Huang, 1985). The primary neurons are therefore one of the earliest differentiating cell groups in the *Xenopus* neural tube. Here we show that co-injecting transcripts encoding both RXR and RAR results in the formation of ectopic primary neurons. Injection of a transcript encoding a dominant negative xRARα2, however, generates embryos that lack primary neurons. These two observations indicate a role for retinoid receptors in primary neurogenesis.

MATERIALS AND METHODS

Isolation of a *Xenopus* RXR cDNA clone

Degenerate oligonucleotide primers (3′ primer 1: 5′-GCYTCNAR-CATYTCCAT-3′; 5′ primer 2: 5′-GGNTGGAAT/CGARYT-3′; 5′ primer 3: 5′-GARTGGGCNAARA/CG-3′) were made against conserved regions of RXR sequences (Mangelsdorf et al., 1992). Primers #1and 3 were used in a PCR reaction to amplify a fragment of approximately 500 bp from a cDNA library in λ phage derived from stage 27 (tailbud) embryos (gift of Dr D. Cleveland). Following reamplification with primer #1 and 5′ primer #2, the fragment was ligated into the TA vector, pCRII (Invitrogen), and RXR clones identified by sequencing with the Sequenase dideoxy protocol (USB). The RXR fragment was then used to screen a stage 24-26 cDNA library (gift of Drs Lemaire and Gurdon) and three clones were identified. The sequence was compiled and analysed using Staden software, and is available on the EMBL database with accession number X87366.

Characterisation of xRXRβ expression

For Northern blots, xRXRβ transcripts were detected with a random-primed probe (Feinberg and Vogelstein, 1983) derived from the entire coding region. For wholemount in situ hybridisation, a DIG-labelled RNA probe was synthesised corresponding to the A/B domains of the protein including the 5′ untranslated region, from bases 1 to 373. This DIG-labelled RNA probe will detect just those transcripts corresponding to the cDNA clone and is therefore unlikely to detect alternatively spliced forms of transcript from the same gene. The corresponding sense probe did not generate a signal (data not shown). Wholemount in situ hybridisation was according to Harland (1991) with minor modifications (see Baker et al., 1995 for details).

In vitro analysis of xRXRβ

The 5′ and 3′ untranslated regions of xRXRβ were replaced with those of the *Xenopus* globin cDNA to generate a construct whose transcripts are efficiently translated. The entire xRXRβ coding region was amplified with primers corresponding to the sequence surrounding the start of translation and the sequence at, and beyond, the termination codon. The latter oligonucleotide was synthesised to included a *Hin*dIII site, and this site was used in combination with the naturally occurring *Nco*I site at the start codon to generate, from the PCR product, a *Hin*dIII-*Nco*I fragment containing the xRXRβ coding sequence. This was cloned into the vector pING14 (gift of S. Ingles and I. Brierley, see also Bannister et al., 1991) at the *Nco*I-*Hin*dIII sites. A similar construct was prepared for the xRARα2 clone (Sharpe, 1992) with the addition of a short sequence encoding a c-myc epitope (Evan et al., 1985) introduced at the 3′ end of the coding sequence by PCR, using oligonucleotides that overlapped the coding sequence of xRARα2, and included the c-myc epitope sequence. Detection of the c-myc epitope has been used to verify and locate the expression of injected xRARα2 in vivo (data not shown).

A control construct encoding a mutated form of xRARα2, called RARα2Δh*d, was generated in two steps. In the first stage, oligonucleotide-directed point mutagenesis was used to introduce a G residue in place of a C residue at position 877 of xRARα2, converting amino acid R103 to G103. This point mutation has been shown to eliminate DNA binding in the related glucocorticoid receptor (Hollenberg and Evans, 1988). In the second step, the putative dimerisation domain (Au-Fliegner et al., 1993) was deleted by linearising the point-mutated clone with *Bgl*II at position 1617 and deleting with *Exo*III. The DNA was religated and the resulting clones sequenced to identify deletions of the required size that remain in frame. RARα2Δh*d contains a deletion from 1575 to 1725 such that D335 is adjacent to T386 (for numbering see Sharpe, 1992).

The dominant negative clone RARαΔ393 was generated by removing a *Sma*I-*Hin*dIII fragment from the carboxy terminus of the xRARα2 clone. This replaces the RAR stop site with that of the globin sequence. As the region around the *Sma*I site is conserved between frogs and higher vertebrates, the deletion breakpoint is identical to that in the dominant negative hRARαΔ403 described previously (Tsai et al., 1992; Damm et al., 1993). The coding region of the original clone in pING14 and the mutated versions were sequenced using the Sequenase (USB) dideoxy protocol. The complete sequence of cDNA clones for xRXRβ (accession number: X87366) and xRARα2 (X87365) are in the EMBL database.

Capped RNA was produced (Krieg and Melton, 1988) from the SP6 promoter of the pING14 vector. Functional viability of transcripts was checked by translation in rabbit reticulocyte lysates followed by bandshift assays (Kliewer et al., 1992) with the programmed lysates and a radiolabelled probe of the retinoic acid response element from the βRAR gene (deThe et al., 1990; Sucov et al., 1990).

Injection of transcripts into embryos

A calibrated needle was used to inject 5-10 nl of synthetic, capped RNA at 50 pg/nl into *Xenopus* embryos using a Medical Systems Corp. picoinjector. For injection, embryos were grown in 1×MBSH, 5% Ficoll. Before gastrulation, embryos were transferred to 0.1×MBSH and grown on to later stages identified by comparison to control embryos at 18° or 23°C. Injected RNAs were detectable by northern blotting until the early tailbud stage and myc-tagged xRARα2 protein was detected by wholemount immunocytochemistry to approximately the same stage (data not shown).

Analysis of injected embryos

Control and injected embryos were analysed by in situ hybridisation (Harland, 1991, with modifications in Baker et al., 1995) using a cDNA probe derived from the XIF3 gene (Sharpe et al., 1989). XIF3 is expressed in primary neurons and neurogenic cranial neural crest. Neural development was also assessed by wholemount immunocytochemistry using the anti-HNK1 monoclonal antibody (Sigma), a peroxidase-conjugated secondary antibody and the Pierce metal-enhanced DAB staining procedure. The anti-HNK1 antibody identifies sensory neurons (Rohan-Beard cells), some interneurons (Kolmer-Agduhr cells), axon tracts, the trigeminal ganglion, a subset of cranial neural crest cells and a small number of cells in the anterior neural tube, and therefore gives the same pattern of staining in *Xenopus* embryos as the monoclonal antibody HNK1 (Nordlander, 1989; Ruiz i Altaba and Jessel, 1991).

RNA encoding β-galactosidase was in some cases co-injected to mark the distribution of introduced RNA. To detect the resultant β-galactosidase activity, embryos were fixed for 1 hour in MEMFA (which contains 4% formaldehyde) and then stained with Xgal according to Turner and Weintraub (1994). Stained embryos were then prepared for wholemount immunocytochemistry with anti-HNK1 and finally embedded in PEDS wax and cut transversely into 12 μm sections.

Animal cap assays

RNA was injected into both cells of the two-cell stage embryo. These were then grown in 0.1× MBSH to the late blastula stage when animal caps were removed and cultured to the tailbud stage in 1× MBSH. Where appropriate, RA in DMSO was added to a final concentration of 10^{-7} M, with the equivalent amount of DMSO alone added to control caps. In each experiment, nucleic acid was extracted from either five animal caps or parts of an embryo or two whole embryos and run on a 1.2% northern blot gel before transfer to Genescreen filters. The cytokeratin probe was an *Eco*RI-*Hin*dIII fragment from the clone CK1(8) (Franz and Franke, 1986).

Inhibition of cell division

Embryos from stage 10, the onset of gastrulation, were maintained in 20 mM hydroxyurea and 150 μM aphidicolin (HUA), which rapidly blocks cell division, as described by Harris and Hartenstein (1991) except that the inhibitors were used in 0.2× MBSH. In this experi-

ment, untreated embryos were grown in 0.2× MBSH supplemented with DMSO to the equivalent amount used as a vehicle for the aphidicolin.

CAT activity assays

Embryos were injected with 15 pg of a plasmid containing the chloramphenicol acetyl transferase gene driven by a thymidine kinase promoter and a retinoic acid response element (a DR-5 derived from the RARβ promoter). This is active in embryos in the absence of RA but is induced to higher levels by the addition of RA (data not shown). Consequently embryos were treated with 10^{-6} M RA at the blastula stage and collected for standard CAT assay at the end of gastrulation. To test the activity of the dominant negative receptor, embryos were coinjected with plasmid and synthetic RNA encoding xRARα2Δ393.

RESULTS

Temporal and spatial expression of xRXRβ during normal development

An xRXRβ cDNA clone was used to determine the timing of xRXRβ expression by hybridisation to a developmental stage series northern blot (Fig. 1). Transcripts found in the four-cell embryo (stage 3) are likely to be maternal as embryonic expression begins at the midblastula transition (stage 8-9) (Newport and Kirschner, 1982). The level of transcripts increases during gastrulation (stages 10-12) and reaches a peak in the early tailbud embryo (stage 22). There is then a marked decrease to a lower level, which is maintained to the swimming tadpole stages. xRXRβ transcripts are therefore most abundant during the neurula stages, the period over which the primary neurons are determined and begin to differentiate (Lamborghini, 1980; Chitnis et al., 1995). Transcript-specific antisense xRXRβ probes were used for whole-mount in situ hybridisation. In the late neurula embryo transcripts are found at a low level in most tissues, but are most abundant in anterior neurectoderm and adjacent cranial neural crest (Fig. 2A). At the early tailbud stage transcripts are detected in the anterior neural tube, eye primordia, and cranial neural crest (Fig. 2B).

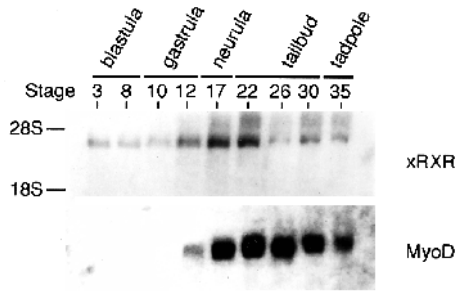

Fig. 1. The peak of xRXRβ expression is during the late gastrula and neurula stages. A northern blot of developmentally staged embryos (Nieuwkoop and Faber, 1994) was assayed with an xRXRβ probe. The xRXRβ transcripts are approximately 3.5 kb long. The same blot was hybridised to an xMyoD probe (Hopwood et al., 1989) as a control for RNA loading and shows the predicted pattern across the developmental series.

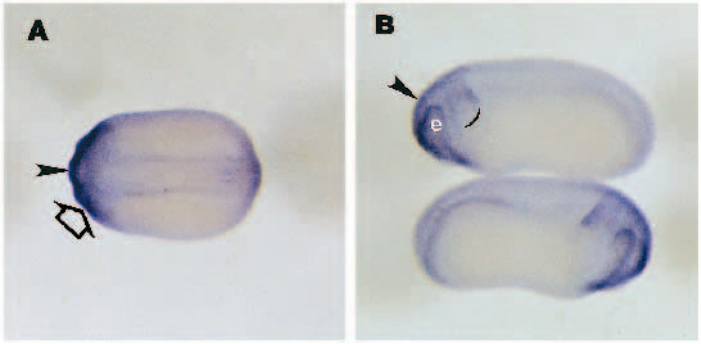

Fig. 2. xRXRβ transcripts are found predominantly in the anterior neurectoderm and adjacent neural crest. A transcript-specific probe derived form the 5′ part of the cDNA clone was used to detect xRXRβ transcripts in the neurula and tailbud embryo. The embryos were rendered transparent in Murray's clear. (A) Dorsal view at the neurula stage shows an accumulation of transcripts in the anterior neurectoderm (arrowhead) and in adjacent neural crest (open arrow), though essentially all tissues express a low level of xRXRβ when determined by RNase protection assay (data not shown). (B) At the tailbud stage transcripts are localised to the anterior neural tube (arrowhead), the dorso-frontal segment of the developing eye (e) and migrating cranial neural crest (bracketed).

Injection of retinoid receptor transcripts into Xenopus embryos

Synthetic RNA was prepared from the coding region of xRXRβ flanked by Xenopus globin non-coding sequences in the vector pING14. A similar construct was made for xRARα2 and, in addition, a control plasmid, xRARα2XΔh*d, was made by introducing a disabling point mutation into the DNA binding domain and a deletion across the heterodimerisation domain such that the xRARα2XΔh*d protein is inactive.

The function of retinoid receptors in neural development was addressed by injecting synthetic mRNA from these constructs into *Xenopus* embryos. As the heterodimer is often the active receptor in retinoid signalling (reviewed in Mangelsdorf and Evans, 1995) and because xRXRβ and xRARα2 can interact in vitro (data not shown), synthetic RNAs encoding xRXRβ and xRARα2 were co-injected into embryos. Injected embryos were assayed by wholemount in situ hybridisation at the tailbud stage using a probe that recognises transcripts from the neural specific XIF3 gene (Sharpe et al., 1989) and also by wholemount immunocytochemistry with the anti-HNK1 antibody.

Co-injection of transcripts encoding xRXRβ and xRARα2 altered the normal pattern of XIF3 expression with embryos developing patches of XIF3 staining adjacent to the neural tube (Fig. 3A) that are likely to represent ectopic neurons. Anti-HNK1 staining confirms this observation, and in addition shows that the ectopic neurons extend axon-like structures (Fig. 3G,H). In comparison, coinjection of xRXRβ with the control xRARα2XΔh*d, or injection of either xRXRβ or xRARα2 alone, had no effect on XIF3 expression (Fig. 3B-F).

xRXRβ/xRARα2 heterodimer overexpression does not induce the expression of XIF3 in isolated animal caps

Given that co-injection of xRXRβ and xRARα2 results in the

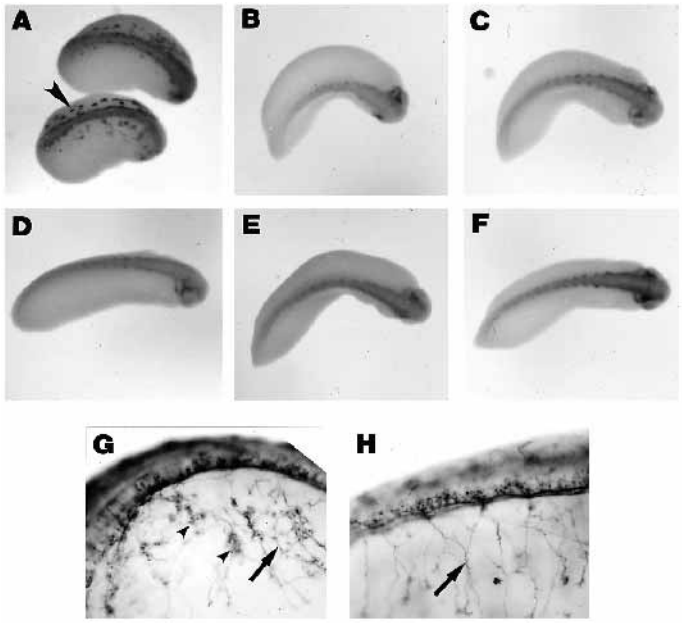

Fig. 3 Co-injection of RNA encoding heterodimeric xRXRβ and xRARα2 results in the formation of ectopic neurons at the tailbud stage. Dorsal views of embryos stained by wholemount in situ hybridisation for XIF3 at the tailbud stage, (anterior to the right). Embryos were injected with: (A) xRARα2/xRXRβ, (B) xRARα2, (C) xRXRβ, (D) xRARα2Δh*d, (E) xRARα2Δh*d/RXRβ; (F) was uninjected. Co-injection of xRARα2/xRXRβ results in embryos that have abnormal patterns of XIF3 expression with positive regions adjacent to the neural tube (e.g. arrow in A). However injection of individual receptors (B,C) or a combination of control transcript xRARα2Δh*d and xRXRβ (E) has no effect on XIF3 expression. Embryos injected in an identical manner were assayed with the anti-HNK1 monoclonal antibody to demonstrate the formation of ectopic neurons. (G) xRARα2/xRXRβ-injected and (H) uninjected embryos rendered transparent in Murray's clear to see neural cell bodies. Axons (arrows) are apparent in both embryos but ectopic clusters of anti-HNK1 positive cell bodies (arrowheads) are only seen adjacent to the neural tube in xRARα2/xRXRβ injected embryos (G) and not in controls (H).

formation of ectopic neurons in whole embryos, we have asked whether this is also sufficient to direct the expression of a neural marker, XIF3, in isolated animal caps that will not receive the normal neural inducing signals. Consequently, blastula-stage animal caps from injected and control embryos were cultured to the tailbud stage in the presence or absence of RA. The northern blot shows that over-expression of retinoid receptors in animal caps is insufficient to cause expression of the neural marker XIF3 (Fig. 4).

The blot was reprobed with a *Xenopus* cytokeratin probe, cyt 1(8) (Franz and Franke, 1986). This showed that each lane contained RNA, but furthermore, that the pattern of cytokeratin expression was altered by the coinjection of RXR/RAR in animal caps that were cultured with RA. It is likely that this change in cytokeratin gene expression is similar to that described in other systems where epidermal cells are treated with RA (Fuchs and Green, 1981; Ferretti et al., 1991). In addition, the change in cytokeratin expression shows that the injected receptors are capable of mediating retinoid signalling but that this signalling alone is insufficient to induce XIF3 expression.

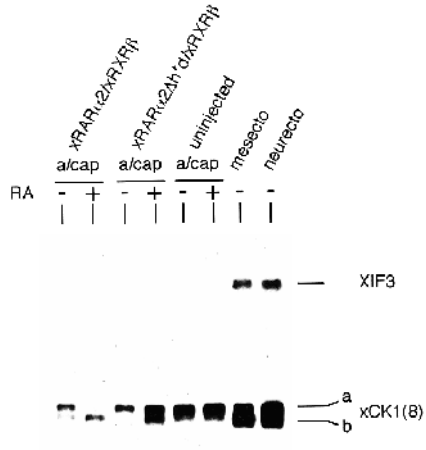

Fig. 4. Injection of xRARα2/xRXRβ does not cause the expression of XIF3 in isolated animal caps. Animal caps (a/caps) were removed from embryos at the late blastula stage. For each batch, half were grown in control media and the other half in medium containing RA. Two positive controls for neural induction were used: firstly,explants consisting of the dorsal lip and a strip of ectoderm (mesecto) were isolated from gastrula embryos and secondly, neural plate neurectoderm (neurecto) was removed from early neurula embryos, and both were grown to the tailbud stage. XIF3 expression is only detected in the positive controls and not in animal caps. Reprobing the northern blot with a probe derived from the cytokeratin 1(8) cDNA clone shows that RA can alter the expression profile of cytokeratin in animal caps derived from xRARα2/xRXRβ-injected embryos. In these animal caps the CK1(8) band labelled 'a' is replaced by a smaller transcript 'b'. This also occurs to a lesser extent in the control xRARα2Δh*d/xRXRβ-injected animal caps, and as a similar result is seen in xRXRβ single-injected animal caps (data not shown), it suggests that RXR homodimers have a reduced ability to cause this effect.

Ectopic neurons still form when injected embryos are treated with a potent inhibitor of cell proliferation

Retinoid signalling affects cell proliferation (Ide and Aono, 1988; Schilthuis et al., 1993) and the increased proliferation of neural precursors could cause an overexpression phenotype. Cell division in *Xenopus* can be rapidly and effectively blocked with a cocktail of hydroxyurea and aphidicoline (HUA) (Harris and Hartenstein, 1991). Consequently we have used HUA treatment to examine the contribution of cell proliferation to the RXR/RAR ectopic neuron phenotype. Uninjected and control xRARα2Δh*d/xRXRβ co-injected embryos remain essentially normal following HUA treatment (Fig. 5). In contrast, embryos co-injected with xRXRβ/xRARα2 at the two-cell stage and grown continuously in HUA from gastrulation (stage 10) again show characteristic ectopic XIF3 staining at the tailbud stage, suggesting that the RXR/RAR phenotype is not due to increased proliferation of neural precursors.

Sensory neurons fail to form in embryos injected with the dominant negative receptor xRARα2Δ393

xRARα2Δ393 has a deletion of the carboxy terminus of the protein from an equivalent position to that introduced into human RARα that confers dominant negative activity (Damm et al., 1993). Three approaches have been taken to confirm that xRARα2Δ393 can act as a dominant negative. Firstly, in vitro-

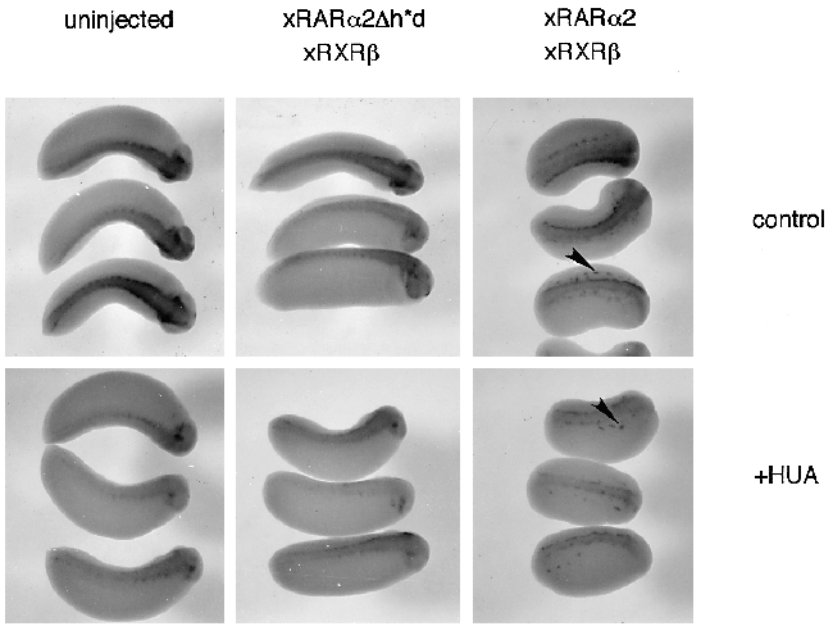

Fig. 5. Ectopic neurons form even when cell proliferation is inhibited in neural precursors. Embryos at the early gastrula stage were treated with hydroxyurea and aphidicolin (HUA) to inhibit cell division (Harris and Hartenstein, 1991) and assayed for XIF3 at the tailbud stage. XIF3 staining is somewhat punctate along the neural tube of uninjected and control xRARα2Δh*d/xRXRβ-coinjected embryos, probably reflecting the HUA treatment. Coinjection of xRARα2/xRXRβ, however, generates ectopic neurons both in untreated and HUA-treated embryos (arrowheads). It is therefore likely that the ectopic neurons do not form through increased proliferation of neural precursors.

translated xRARα2Δ393 retains the ability to heterodimerise and bind to a retinoic acid receptor element (RARE) in a band shift assay (Fig. 6A). Secondly, xRARα2Δ393 transcripts injected into embryos repress transcription from an introduced RARE-tk-CAT reporter gene in a dose-dependent manner (Fig. 6B). Finally, the change of cytokeratin expression in xRARα2/xRXRβ-injected animal caps in response to added RA, as described above, can be reversed by the injection of increasing amounts of dominant negative receptor xRARα2Δ393 transcripts (Fig. 6C).

During normal development, the primary neurons begin to function in the tailbud embryo controlling the ‘sideways-flip’ movements that can be elicited in response to stimulation. Embryos injected solely with xRARα2Δ393 mRNA failed to show these movements and a lack of sensory neuron axons along their flanks, demonstrated by wholemount immunocytochemistry with anti-HNK1 (Fig. 7A-C), is likely to contribute to this defect.

Injection into one cell of a two-cell embryo places injected transcripts on just one side of the later embryo. Embryos injected in this way with xRARα2Δ393 mRNA lack primary sensory neurons (Rohan-Beard cells) only on the injected side of the embryo (Fig. 7D,E). Although it is clear that sensory neurons fail to form, analysis of the effects on interneuron and motor neuron formation will require probes that discern individual cell types. However, a class of interneurons (Kolmer-Agduhr cells) detected by the anti-HNK1 antibody are consistently unaffected by this treatment, suggesting that not all

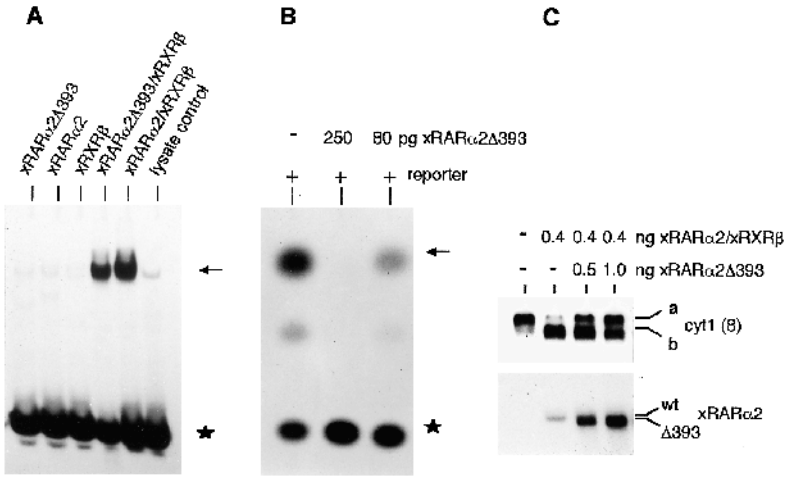

Fig. 6. xRARα2Δ393 acts as a dominant negative inhibitor. (A) Rabbit reticulocyte lysates producing either xRXRβ, xRARα2 or xRARα2Δ393 were used in a band shift assay with a βRARE oligonucleotide probe. Lysates containing a single receptor type did not shift the probe above background levels (compare the unprogrammed lysate control). However mixed lysates producing xRARα2 and xRXRβ, or xRARα2 and xRARα2Δ393, retarded much larger amounts of probe, showing that xRARα2Δ393 retains its ability to dimerise with xRXRβ and bind to DNA. Arrow, band-shifted probe; asterisk, unshifted probe. (B) *Xenopus* embryos can drive expression of a CAT reporter gene from a βRARE enhancer and a thymidine kinase (tk) promoter. However coinjection of the reporter construct and transcripts encoding xRARα2Δ393 results in a dose-dependent inhibition of transcription, indicating that xRARα2Δ393 represses endogenous retinoid signalling through the βRARE-tk promoter. (C) Embryos were injected at the two-cell stage and animal caps removed from late blastula embryos and grown to the tailbud stage. Two embryos from each batch were kept whole and collected at the late gastrula stage to analyse injected RNA. As seen in Fig. 5, the cytokeratin expression profile in xRARα/xRXRβ-injected animal caps is altered in the presence of RA from band ‘a’ to band ‘b’. Coinjection of the transcript encoding the dominant negative antagonises the effect of xRARα/xRXRβ, increasing the proportion of band ‘a’. The northern blot of gastrula stage embryos does not resolve wild-type and dominant negative transcripts but shows the lack of endogenous transcripts in the uninjected animal caps and the increasing levels of transcript in the experimental animal caps.

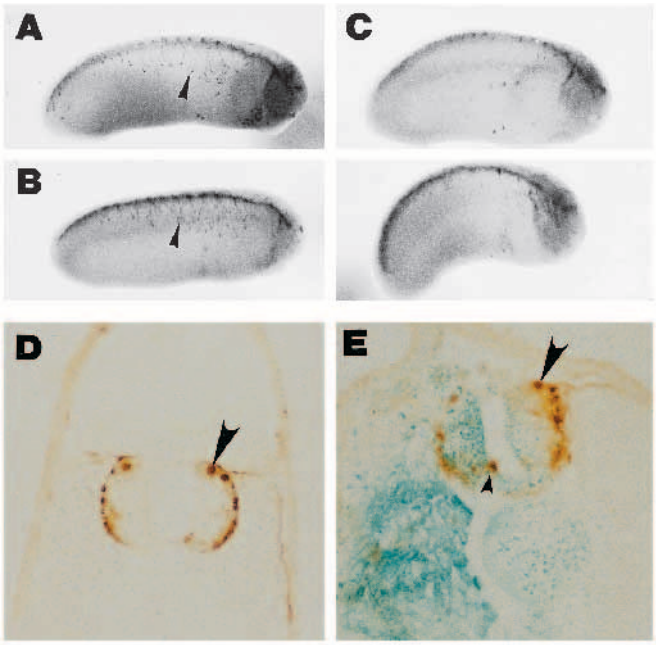

Fig. 7. Injection of transcripts encoding the dominant negative xRARα2 result in a lack of sensory neurons. (A,B) Embryos injected with the dominant negative xRARα2Δ393 into both cells at the two-cell stage have decreased numbers of sensory neuron axons, detected by the anti-HNK-1 antibody. (A) Uninjected embryo at the tailbud stage. Examples of sensory neuron axons are marked by arrowheads. (B) Embryo injected with control transcript xRARα2Δh*d has the normal complement of axons. (C) Embryos injected with 400 pg of xRARα2Δ393 have very few sensory axons. Staining within the neural tube represents the cell bodies and axon tracts of anti-HNK-1 positive cells that are unaffected by xRARα2Δ393 transcript injection. (D) Transverse section through an uninjected embryo showing bilateral primary sensory neurons (large arrowhead) and axons. (E) Transverse sections through a tailbud stage embryo co-injected into one cell at the two-cell stage with xRARα2Δ393 and synthetic RNA encoding β-galactosidase. At the tailbud stage the embryos were assayed for both β-galactosidase activity (blue stain) and with the anti HNK-1 antibody to detect sensory neurons (brown). β-galactosidase activity acts as a lineage tracer to identify those parts of the embryo that received the coinjected synthetic transcripts. Only regions that did not receive injected transcripts developed primary sensory neurons (large arrowhead), although anti-HNK-1 staining is still seen in axon tracts and in some interneurons (small arrowhead). In addition, the ventral neurocoel is consistently displaced towards the uninjected side of the embryo.

primary neurons fail to differentiate in response to the expression of the dominant negative RAR. In addition xRARα2Δ393 does not inhibit neural tube formation, most cells of which will contribute to the secondary or 'adult' nervous system. However, xRARα2Δ393 injection does alter neural tube morphology in the spinal cord, displacing the ventral neurocoel towards the uninjected side of the embryo.

In summary, we have used two contra-acting manipulations, the provision of additional receptors and the injection of a dominant negative, to examine retinoid receptor function. From this a consistent picture emerges in which over-expression of wild-type receptors results in the formation of ectopic primary neurons whilst the dominant negative generates a neural tube lacking primary neurons.

DISCUSSION

We are interested in examining the role of retinoid signalling, particularly the contribution of retinoid receptors to neural development in *Xenopus*. Previous analyses have concentrated on the expression of xRXRα and xRXRγ; however these clones are not extensively expressed in the gastrula and neurula stages (Blumberg et al., 1992) when xRXRβ in combination with either xRARα2 or xRARγ is likely to form active heterodimeric receptors. As xRARα2 is expressed throughout the embryo, with elevated levels in the neurectoderm, whilst xRARγ is restricted to anterior and posterior expression domains, we manipulated the expression of xRXRβ and xRARα2 and examined the subsequent effects on neural development.

Injection of xRXRβ/xRARα2 produces ectopic neurons

Overexpression of xRXRβ/xRARα2 heterodimers results in the appearance of neurons in a region adjacent to the neural tube. Their identity as neural cells is established by two neural markers, the expression of XIF3 and cross-reactivity to monoclonal antibody anti-HNK 1.

Retinoid signalling can affect cell proliferation, as signalling through RARα1 mediates growth inhibition in a newt limb blastemal cell line (Schilthuis et al., 1993) whilst retinoic acid promotes proliferation of the distal mesodermal cells of the chick limb bud (Ide and Aono, 1988). To examine whether enhanced proliferation is the cause of ectopic neuron formation, xRARα2/xRXRβ co-injected embryos were treated with hydroxyurea and aphidicoline. This causes a rapid inhibition of cell division but has little effect on development and differentiation up to the tailbud stage (Harris and Hartenstein, 1991). This approach has been successfully used to show that cell proliferation plays no role in the neural phenotypes observed following injection of XASH-3 (Turner and Weintraub, 1994) and xDelta (Chitnis et al., 1995). HUA treatment does not inhibit the formation of ectopic neurons in xRARα/xRXRβ-injected embryos, suggesting that receptor over-expression does not result in an increased rate of neural precursor proliferation. The ectopic neurons are therefore likely to arise either through a change in cell fate or by altered cell migration. An examination of the development of the ectopic neuron phenotype at earlier stages may help to resolve this point.

Although we cannot excluded the possibility that injection of a single receptor type may affect aspects of development for which we have not assayed, ectopic neurons were only seen when transcripts encoding both xRXRβ and xRARα2 were injected. This suggests that the effect depends on the formation of heterodimers in the embryo. The activity of injected xRXRβ and xRARα2 transcripts is reminiscent of the enhanced activity of *Xenopus achaete-scute* homologue 3 (XASH-3) following co-injection with its heterodimeric partner E12 (Ferreiro et al., 1994). Although the ectopic neuron phenotype depends on the injection of the heterodimer, the precise mechanism is uncertain. One possibility is that additional receptors enhance signalling from endogenous retinoids, although the phenotype might equally be due to the repressive effects of unliganded receptor dimers. The recent discovery of cofactor proteins that bind to receptor dimers and repress transactivation in the

absence of ligand (Chen and Evans, 1995; Hörlein et al., 1995) raises the possibility that the phenotype could arise from the titration of these factors leading to activation of transcription. Both RXR and RAR are encoded by three different genes, each of which can generate more than one protein. An extension of this analysis to other combinations of xRXR and xRAR may contribute to an understanding of the mechanism by which ectopic neurons are generated. It is emphasised however that we have concentrated on the effects of injecting xRXRβ and xRARα2, as these are present in the neurectoderm during neurogenesis.

The phenotype following injection of a dominant negative xRARα2

xRARα2 has been truncated at the carboxy-terminal domain to produce a protein that lacks a transcriptional transactivation domain but still heterodimerises with xRXRβ and binds DNA (Fig. 6A). Due to sequence conservation, the deletion begins at the same *Sma*I site used in the creation of RARαΔ403, a dominant negative mutation of hRARα (Damm et al., 1993). Transgenic mice with widespread expression of RARαΔ403 have developmental defects, including a cleft palate (Damm et al., 1993), though an analysis of the nervous system has not been described. Directed expression of RARαΔ403 in the epidermis of transgenic mice reveals a role for retinoid signalling in epidermal maturation (Imakado et al., 1995; Saitou et al., 1995), whilst expression in a multipotent hematopoeitic cell line both inhibits self-renewal and promotes differentiation towards basophils and mast cells as opposed to their normal neutrophil or monocyte fate (Tsai et al., 1992). As carboxy-terminal-deleted dominant negative retinoid receptors may act by losing their ability to dissociate repressors in the presence of RA, the identical deletion site in xRARα2Δ393 and RARαΔ403 points towards xRARα2Δ393 similarly acting as a dominant negative receptor. In support of this we have shown that xRARα2Δ393 represses transcription from a reporter gene driven by an RA-dependent promoter in *Xenopus* embryos (Fig. 6B), and antagonises an RA-dependent change in cytokeratin expression in animal caps from xRARα2/xRXRβ injected embryos (Fig. 6C).

Whilst embryos injected with the dominant negative xRARαΔ393 look outwardly normal, they are clearly distinct from control embryos in their inability to respond to physical stimulation with a tailflip response. Injection of a dominant negative form of xRARγ (Smith et al., 1994) does not cause a phenotype. However, injecting v-erb-A, a mutated form of the chick thyroid hormone receptor α, interferes with retinoid signalling and generates *Xenopus* embryos with cranial crest defects (Schuh et al., 1993). In both the above cases, the development of the nervous system was not specifically examined.

As a result of an interaction with RXR, native thyroid hormone receptor can suppress RAR-dependent transactivation by sequestering RXR (Barettino et al., 1993). The lack of sensory neurons following xRARα2Δ393 injection could therefore result from the sequestration of available RXR that would normally bind to other nuclear hormone receptors. On these grounds one might predict that the same phenotype would follow the injection of normal xRARα2, which in fact has little or no effect on neural development (Fig. 3). It is therefore unlikely that the lack of neurons phenotype is due to the inhibition of a second non-retinoid signalling pathway through the sequestration of RXR vital to each pathway.

In the mouse, individual retinoid receptor isoforms have been eliminated by homologous recombination, for the most part without a major effect on development and differentiation (for example: Li et al., 1993; reviewed in Kastner et al., 1995). Where more than one isoform has been mutated the mice may show defects often, but not entirely, corresponding to those seen in the vitamin A deficiency syndromes. However defects in the differentiation of cells within the neural tube have not been reported. This may reflect the need to totally eliminate retinoid signalling, which may occur with a dominant negative but is unlikely in mutated mice where other members of the gene family may have sufficient activity to compensate for the loss (Kastner et al., 1995).

Nuclear hormone receptors and neural development

The *Xenopus* nervous system probably forms in response to a series of events. Initially, during induction, factors such as noggin (Lamb et al., 1993) and chordin (Sasai et al., 1995) are involved in committing cells to a neural fate. Subsequently groups of cells within the neurectoderm are selected to form primary neurons in a process that involves expression from the *Xenopus Notch* and *Delta* genes (Chitnis et al., 1995) and possibly the *achaete-scute* homologue XASH3 (Turner and Weintraub, 1994; Ferreiro et al., 1994). In addition, genes such as *NeuroD* (Lee et al., 1995) are required for neuronal differentiation. Imposed on this series of events is the process of patterning both along the anterior-posterior and dorsal-ventral axes. RA has been shown to influence patterning (e.g. Marshall et al., 1992), and certain genes involved in this process possess RA response elements (e.g. Marshall et al., 1994). However, the evidence that RA signalling is involved specifically in neurogenesis comes mainly from the analysis of pluripotential embryonal carcinoma (EC) cell lines that form neural derivatives in response to RA (McBurney et al., 1988). Furthermore, the loss of this ability in the P19 RA C65 EC cell line can in part be attributed to its expression of a carboxy-terminal-deleted form of RARα that acts in vitro as a dominant negative (Pratt et al., 1990). The animal cap experiments reported here show that retinoid signalling alone is insufficient to generate the expression of XIF3, a marker of primary neural differentiation. However, overexpression of *xNotch* and *xDelta* are likewise unable to direct animal caps to a neural fate, acting as they do downstream of the initial induction process.

The *staggerer* mutation in mice, characterised by a defect in the development of cerebellar Purkinje cells, is due to a lesion in the RORα gene (Hamilton et al., 1996), whilst *eagle,* expressed in a subset of *Drosophila* neuroblasts, is involved in regulating the fate of neurons derived from these cells (Higashijima et al., 1996). Both RORα and *eagle* encode nuclear hormone receptors. Combined with the results presented here, one may predict a wider role for nuclear hormone receptors in neural development.

In summary, overexpression of retinoid receptors results in the formation of ectopic neurons whilst the dominant negative results in a ‘lack of neurons’ phenotype. These contra-acting manipulations suggest that retinoid signalling is involved in the formation or differentiation of primary neurons in addition to its proposed role in axial patterning.

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