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An essential role for retinoid signaling in anteroposterior neural patterning

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SUMMARY

The vertebrate central nervous system (CNS) is induced by signals emanating from the dorsal mesoderm, or organizer, that divert the ectoderm away from an epidermal and towards a neural fate. Additional signals from the organizer pattern the neural ectoderm along the anteroposterior axis. We devised highly specific methods utilizing constitutively active or dominant negative receptors to evaluate the role of retinoids in neural patterning. Microinjection of these reagents either augments or reduces retinoid signaling in specific regions of the embryo. We show that increased receptor activity suppresses anterior neural structures while dominant negative receptors lead to anterior enhancement. Similarly, microinjection of the dominant negative receptor leads to the loss of posterior marker genes. We demonstrate that retinoid receptors comprise a critical component in neural posteriorization and are required for proper neuronal differentiation. These results support a quantitative role for retinoid signaling in regionalization of the CNS.

Key words: A/P neural patterning, RAR, Retinoid, Dominant negative

INTRODUCTION

Vitamin A and its congeners such as retinoic acid (RA) contribute to diverse aspects of development of the heart, respiratory tract, eye, epithelia and other embryonic structures suggesting its involvement in their normal development (Sporn et al., 1994). Biological effects of RA are primarily mediated through the action of two classes of specific receptors, RARS and RXRs (Mangelsdorf et al., 1994; Kastner et al., 1995). Both classes are members of a large superfamily of ligand-activated transcription factors, the nuclear receptors (reviewed by Mangelsdorf et al., 1994; Kastner et al., 1995). RARs are activated by all-*trans* and 9-*cis* RA, RXRs are specifically activated by 9-*cis* RA (Heyman et al., 1992; Levin et al., 1992). Many aspects of vitamin A deficiency syndrome were recapitulated by knockouts of RARs and RXRs in various combinations (Mangelsdorf et al., 1994; Kastner et al., 1995) implicating retinoid signaling though these receptors as key regulators of early development.

One regulatory role suggested for RA is in the developing nervous system. RA was first proposed to be involved in neural development when it was demonstrated that exogenously applied RA produces a concentration-dependent truncation of anterior, and enhancement of posterior structures in *Xenopus* embryos (Durston et al., 1989; Sive et al., 1990) through its influence on the embryonic mesoderm and ectoderm (Ruiz i Altaba and Jessell, 1991; Sive and Cheng, 1991). At low concentrations, RA suppresses anterior development within the hindbrain while at high concentrations it causes more global anterior truncations, reflected by suppression of anterior markers such as Otx (Durston et al., 1989; Papalopulu et al., 1991a; Papalopulu and Kintner, 1996; see also review by Conlon, 1995 and references therein). Sharpe (1991) showed that retinoic acid (RA) could also mimic the action of endogenous signals involved in inducing posterior gene expression in the *Xenopus* nervous system. The actual role of RA in development remains controversial since evidence for its involvement in normal processes is primarily based on ‘gain of function’ experiments involving addition of exogenous RA.

To overcome these problems and understand RA-mediated signaling during early *Xenopus* development, especially its potential role in neural patterning, we investigated the effects of locally altering RAR signaling. First, by microinjecting a constitutively active receptor, we mimicked local increases in retinoid concentration and uncoupled the transcriptional effects of retinoids, mediated through nuclear receptors, from potentially less specific effects on cellular binding proteins, cell membranes or viability. Second, by microinjecting a dominant negative xRARα that inhibited RAR signaling, we evaluated whether activation of these receptors by endogenous retinoids has a role in normal development. Our approach allowed the

evaluation of dominant gain or loss of RAR activation and provided an opportunity to investigate these effects in vivo, using intact embryos as opposed to using in vitro explant and transplantation experiments as in prior studies.

MATERIALS AND METHODS

Construction of xRAR expression plasmids

We and others previously isolated *Xenopus* RARs (xRARα1,α2 and xRARγ1,γ2) and RXRs (xRXRα and xRXRγ) (Blumberg et al., 1992; Ellinger-Ziegelbauer and Dreyer, 1991; Sharpe, 1992). In preliminary experiments we determined that retinoids selective for the RAR pathway, and particularly for xRARα, were the most potent teratogens when applied during early development (data not shown).

xRARα2 was isolated from a *Xenopus* gastrula cDNA library (Cho et al., 1991) using xRARα1 as a probe. DNA sequence analysis showed that it is essentially identical to xRARα2.1 (Sharpe, 1992). The protein coding regions of xRARα cDNAs were cloned into the *Nco*I-*Bam*HI sites of the vector pCDG1 (Blumberg et al., 1996) after introduction of these restriction sites into the cDNAs by site directed mutagenesis. VP16-xRARs were constructed by cloning the VP16 activation domain (amino acids 1-78) into pCDG-xRARα1 or α2 between the β-globin 5′ leader and the xRARα coding regions. xRARα deletion constructs were produced by exonuclease III digestion of cDNAs followed by recircularization.

Transfections

For evaluation of response to RA, the pCDG1-xRARα expression constructs were co-transfected into CV-1 cells with the reporter gene tk-(βRARE)$_2$-luc and CMX-β-galactosidase control plasmids (Perlmann et al., 1993). Transfections, ligand addition, luciferase and β-galactosidase assays were performed in triplicate using a robotic microassay system as described (Blumberg et al., 1996). The ability of the constructs to activate or repress reporter gene activity was tested over a range of RA concentrations from 10^{-11} M to 10^{-5} M. The results depicted in Fig. 1B show the result at 10^{-7} M RA and represent the average of triplicate experiments.

Electrophoretic mobility shift assays were exactly as described using in vitro transcribed, translated xRARs, xRXRα, and the RARβ retinoic acid response element (Perlmann et al., 1993).

Microinjections

mRNA was produced in vitro by linearizing pCDG-xRAR constructs with *Not*I and transcription with bacteriophage T7 RNA polymerase using the mMessage machine kit (Ambion). After template digestion with DNAse I, the RNA was purified by two precipitations from 2.5 M LiCl, resuspended in distilled water at 1 mg/ml and stored at −70°C. For the assays shown in Fig. 1C, 1 ng of mRNA and 100 pg of reporter plasmid (tk-(βRARE)$_2$-luc) were injected bilaterally into two-cell embryos using a Narishige IM-200 microinjector. RA treatment was at 10^{-7} M from stage 8 to stage 11 (Nieuwkoop and Faber, 1967) at 19°C. Embryos were harvested at stage 13 and lysed in 0.1 M KPO_4, 1 mM dithiothreitol, by three cycles of freeze thawing. After brief centrifugation, 20 μl of extract was assayed for luciferase activity and 5 μl for determination of protein concentration. Reporter gene activity was normalized to protein concentration. Embryos evaluated for morphological response to RA treatment after receptor microinjection were microinjected with 1 ng of mRNA, treated with retinoids or solvent controls from stage 8 to stage 11, washed and allowed to develop until stage 42 before fixation.

Whole-mount in situ hybridization

1 ng of receptor and 0.2 ng of β-galactosidase mRNAs were microinjected unilaterally into two-cell albino embryos. Embryos were incubated until stage 16-18 when they were dechorionated and fixed in MEMFA for one hour (Harland, 1991). After rinsing in 0.1 M $NaPO_4$, pH 6.3, the embryos were incubated with X-gal (Coffman et al., 1993) until staining was apparent, typically 1-2 hours. After a brief rinse in water, the embryos were transferred into 100% ethanol and stored at −20°C. In situ hybridization was performed essentially as described (Harland, 1991). Antisense probes were prepared using digoxygenin-11-UTP (Boehringer Manheim) using the Megascript system (Ambion). After synthesis and DNAse removal of plasmid templates, RNAs were purified by two sequential precipitations from 2.5 M LiCl and stored in hybridization buffer at 100 μg/ml (Harland, 1991).

RESULTS

The modular nature of nuclear hormone receptors allowed us to construct mutant receptors with specific transcriptional effects. A constitutively active receptor was created by fusing the strong transactivation domain from the herpes simplex virus protein VP-16 (Sadowski et al., 1988) to the amino terminus of xRARα1 (VP-xRARα1) (Fig. 1A). It was previously shown that specific deletions in the RAR ligand binding domain which remove the activation function can result in receptors which dominantly repress transcription from reporter genes containing a RA response element (Damm et al., 1993). These truncated receptors retain the ability to bind ligand, bind DNA and heterodimerize with RXR but cannot activate transcription of target genes in response to RA (Damm et al., 1993). Thus, to make receptors with dominant negative activity, we prepared a series of carboxyl terminal truncations in xRARα1 and tested each for transcriptional activity in transfected CV-1 cells. The strongest repressor of RA signaling was a mutant deleted at amino acid 405 (xRARα1^{405*}) (Fig. 1B,C and data not shown). In addition to repressing normal RA signaling in transfected cells (Fig. 1B) and microinjected embryos (Fig. 1C), xRARα1^{405*} was able to block the teratogenic effects of exogenously applied RA as would be expected for a dominant negative receptor (Fig. 1D). This is in agreement with results from other groups who demonstrated that reagents which interfere with retinoid signaling also impair RA-mediated teratogenesis (Smith et al., 1994; Kolm and Sive, 1995; Schuh et al., 1993; Schuh and Kimelman, 1995). In contrast, the inactive receptor was neutral while the wild-type xRARα1 augmented RA teratogenesis (Fig. 1D) as does xRARγ (Smith et al., 1994).

It is important to note that xRARα1^{405*} functions as a dominant transcriptional repressor which is distinct from those transcriptionally inactive receptors that can bind ligand but not DNA (Smith et al., 1994) and those that can heterodimerize with, and thus sequester RXR such as v-erbA or COUP-TF (Kolm and Sive, 1995; Schuh et al., 1993; Schuh and Kimelman, 1995). In contrast, xRARα1^{405*} binds target sequences (Fig. 1E) where its constitutive transcriptional repression is neither rescued by retinoids or wild-type receptor. Injection of the wild-type xRARα1 gave milder versions of the dominant negative phenotype and did not rescue the dominant negative phenotype when injected at up to two-fold excess over the dominant negative receptor (data not shown). Although all

of the dominant negative data shown derives from injections of xRARα1^{405*} mRNA, microinjection of the equivalent xRARα2^{410*} mutation gave similar results. Our results do not distinguish whether the dominant negative receptors inhibit only xRARα signaling or whether they are capable of influencing activation of xRARγ1 and γ2 which are also expressed anteriorly.

To evaluate the effects of mutant receptor mRNAs on development, embryos were microinjected bilaterally at the two-cell stage and allowed to develop until the tadpole stage (stage 38). Microinjection of the constitutively active VP-xRARα1 mRNA phenocopied RA treatment (Fig. 2C,D) in that anterior structures such as cement gland and eyes were greatly reduced or absent. Microinjection of the dominant negative mutant, xRARα1^{405*}, gave an opposite effect. Embryos showed enlarged anteriors, shorter tails and reduced overall length (compare Fig. 2A and B). Embryos microinjected with mRNAs encoding β-galactosidase, β-globin, or the inactive receptor were morphologically normal (data not shown). Embryos injected with the VP-16 activation domain alone were normal, thus eliminating non-specific transcriptional squelching as a potential teratogenic mechanism (data not shown). Overall, embryos injected with the dominant negative receptor appear anteriorized whereas those injected with the constitutively active receptor appear posteriorized.

These results prompted us to examine whether locally increasing or decreasing xRAR signaling could cause coordinate shifts in the expression of A/P marker genes. This was achieved by microinjecting albino embryos unilaterally at the two-cell stage with 1 ng of receptor mRNA and 0.2 ng of β-galactosidase lineage tracer mRNA. The injected embryos were fixed at stage 16-18 and first stained for β-galactosidase activity which was taken as a measure of injected mRNA localization. Embryos with unilateral β-galactosidase staining were selected, sorted according to where staining was located and in situ hybridization was performed with marker genes expressed in the same area. This enabled us to directly compare effects elicited by the injected mRNA with the normal marker expression in the uninjected contralateral side, thus each injected embryo is internally controlled. For markers within an affected domain, we anticipated the level or extent of expression to be increased or decreased. Markers adjacent to an affected region, were expected to shift along the anteroposterior axis relative to the control contralateral side.

The alterations in gene expression elicited by microinjection of the constitutively active receptor are consistent with those observed after RA treatment of whole embryos. For example, in the neural plate the levels and extent of Otx2 expression were decreased (Fig. 3D,H) while En-2 and Krox-20 were shifted anteriorly (Fig. 3L,P). In about 50% of the injected embryos En-2 and Krox-20 expression was almost completely eliminated, similar to the results obtained after local application of RA (Drysdale and Crawford, 1994). In contrast, the constitutively active receptor had no effect on the normal expression patterns of the posterior markers, Hox-B9 (Fig. 3T) and Xlim-1 (not shown). Thus while it can inhibit the development of anterior tissues, the constitutively active receptor does not further enhance a fully activated posterior program. One possibility is that since posterior tissues have high retinoid levels (Chen et al., 1994), posterior markers are already maximally induced. This interpretation is consistent with the observation that, in the embryo, Hox-B9 is less sensitive to RA addition than more anterior Hox genes (Papalopulu et al., 1991b; Dekker et al., 1992; Conlon and Rossant, 1992). The finding that Otx-2 expression was reduced, but not eliminated, by constitutively active receptor was surprising since exogenous RA strongly suppresses Otx expression (Papalopulu and Kintner, 1996; Simeone et al., 1995; and data not shown). This implies that the teratogenic effects of RA on the forebrain are not mediated solely by xRARα, but might involve other RA dependent pathways (e.g. RXR).

Consistent with the results in Fig. 2, interference with xRAR signaling by the dominant negative receptor led to an increase in the anterior and lateral expression domains of the forebrain/anterior midbrain marker Otx2 and a shift of the expression boundary posteriorly (Fig. 3C,G). En-2, a marker of the mid/hindbrain boundary, is also shifted posteriorly on the injected side (Fig. 3K) indicating an expansion of anterior structures.

Posteriorly, decreased RA signaling led to loss of one of the two Krox-20 stripes, which normally mark rhombomeres 3 and 5 in the hindbrain (Fig. 3O). In *Xenopus*, r5 produces neural crest that migrates laterally, such that the Krox-20 staining in r5 extends more laterally than in the r3 stripe (Bradley et al., 1993). Based on this criterion, it appears that embryos injected with the dominant negative receptor lose the more posterior rhombomere 5. The expression of Xlim-1, a marker of posterior neural plate and lateral plate mesoderm (Taira et al., 1992) is ablated in the injected region (Fig. 4C,D,G,H). Similarly, the expression of Hox-B9, which marks the spinal cord (Wright et al., 1990) is strongly downregulated (Fig. 3S).

It was previously shown that RA treatment could alter neural differentiation of embryonal carcinoma cells (reviewed by Maden and Holder, 1992). Treatment with RA induces the differentiation of P19 teratocarcinoma cells to neurons and glia (Jones-Villeneuve et al., 1982; McBurney et al., 1982) and causes cultured neuroblastoma cells to produce structures resembling dendrites (Sidell, 1982). Furthermore, RA is involved in promoting neuronal differentiation in animal caps neuralised in vitro by injection of noggin mRNA (Papalopulu and Kinter, 1996). These data suggested a role for retinoids in neuronal differentiation and prompted us to ask whether interference with RAR signaling could influence neural differentiation in embryos. N-tubulin is a molecular marker for primary neurons, which differentiate in three stripes on either side of the dorsal midline at the neural plate stage and later give rise to sensory, motor and interneurons (Chitnis et al., 1995). Injection of the dominant negative RAR led to reduction or ablation of N-tubulin positive cells (Fig. 4K,L). Injected embryos which were allowed to develop until the tadpole stage were partially or completely unresponsive to touch stimuli, suggesting an important role for retinoids in neural differentiation as well as A/P specification. Retinoid signaling is involved in the normal differentiation of all three major classes of neurons since all three stripes of N-tubulin are ablated by the dominant negative RAR (Fig. 4K,L).

Fig. 1. Activity of mutant xRARs in vitro and in vivo. (A) Schematic diagram of xRARα expression constructs utilized in this study. (B) Effect of wild-type and mutant xRARαs on reporter gene response to all-*trans* RA in transfected CV-1 cells. (C) Effect of wild-type and mutant xRARs on reporter gene response to all-*trans* RA in microinjected embryos. (D) Wild-type and mutant xRARs alter the morphological response to exogenous all-*trans* RA in microinjected *Xenopus* embryos. (E) Wild-type (xRARα1) and dominant negative RARs (xRARα1^{405*}) bind a retinoic acid response element whereas the control construct (xRARα2^{386*}) does not.

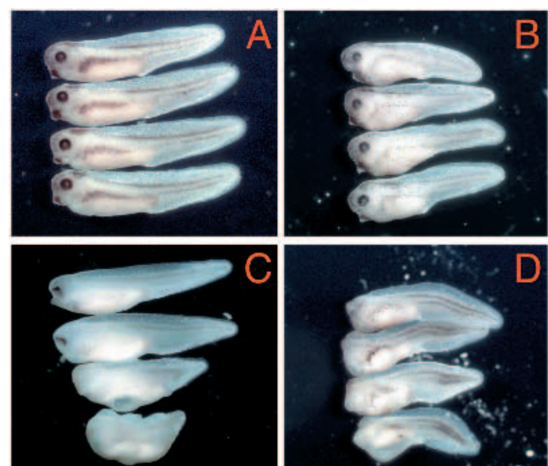

Fig. 2. Constitutively active and dominant negative xRARα have apparently opposite effects on embryonic pattern formation. Wild-type and mutant xRAR mRNAs were evaluated for their ability to alter morphology along the A/P axis. Injections or treatments were as follows: (A) uninjected, (B) xRARα1^{405*}, (C) VP16-xRARα1, (D) 10^{-7} M tRA. mRNAs were injected bilaterally at the two-cell stage and the embryos allowed to develop until fixation at stage 38-40. Microinjections were performed with both α1 and α2 dominant negative receptors. The strongest and most consistent phenotypes were elicited by xRARα1^{405*} and this receptor was utilized for subsequent studies.

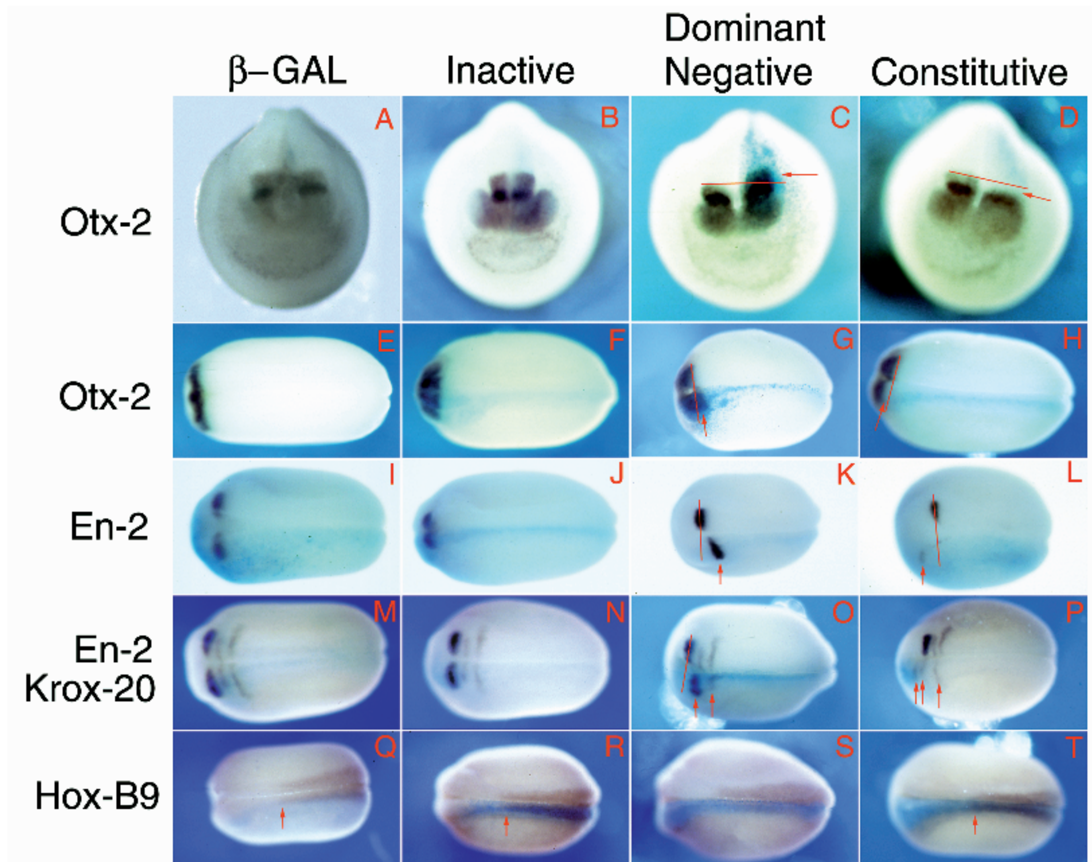

Fig. 3. Mutant xRARα mRNAs alter patterning along the anteroposterior axis. Position specific molecular markers were employed to evaluate the effects of xRARα mRNA injection on A/P neural specification. In situ hybridization analysis of stage 16-18 embryos. In each panel the staining pattern on the injected side should be compared with that on the control, uninjected side. (A-D) The injected side, marked by X-gal staining, is oriented to the right, in all other panels the injected side is on the bottom. (A-H) Xotx-2 (Blitz and Cho, 1995), (I-L) Xen-2 (Hemmati-Brivanlou and Harland, 1989), (M-P) Xen-2 and xKrox-20 (Bradley et al., 1993), (Q-T) Hox-B9 (Wright et al., 1990). (C,D,G,H) The line is oriented at the posterior boundary of Otx-2 staining, the arrow indicates regions of increased (C,G) or decreased (D,H) Otx-2 staining. (K,L) The line is parallel with the anterior edge of the neural folds, arrows indicate the location of En-2 staining. (O,P) The line is parallel with the anterior edge of the neural folds, arrows indicate En-2 and Krox-20 staining on the injected side. (Q-T) Arrows indicate the anterior border of Hox-B9 staining on the injected side, the lack of an arrow in S indicates no staining. Note that the β-galactosidase injected embryos in A,E,J,M,Q are younger than the others resulting in a broader neural plate, however, there is no difference in marker gene expression between the injected and uninjected sides of the embryo.

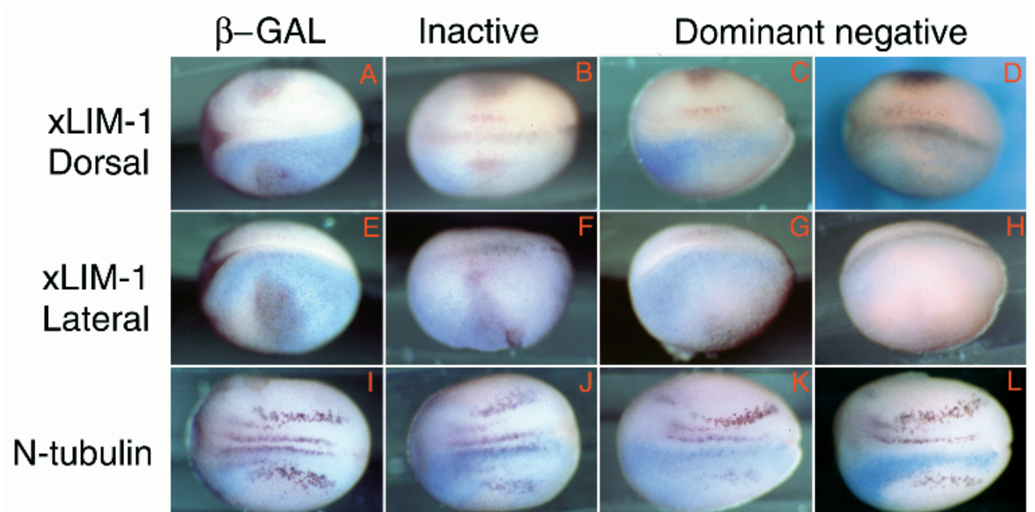

Fig. 4. Retinoid signaling is required for neuronal differentiation. Markers of both neural position and neuronal differentiation were employed to evaluate requirements for xRAR signaling. In situ hybridization analysis of stage 16 embryos (A-H) Xlim-1 (Taira et al., 1992), (I-L) N-tubulin (Chitnis et al., 1995).

DISCUSSION

What role do retinoids play in regionalization of the central nervous system? The currently favored model for neural induction is the two-step 'activation-transformation' model in which the ectoderm is first 'activated' to form anterior neural tissue and then 'transformed' to produce the full spectrum of anteroposterior structures (Nieuwkoop, 1952; Eyal-Giladi, 1954; Gilbert and Saxen, 1993). This model is supported by the observation that all direct neural inducers described to date induce anterior neural tissue (Lamb et al., 1993; Sasai et al., 1995; Hemmati-Brivanlou et al., 1994; Hemmati-Brivanlou and Melton, 1994; Hawley et al., 1995), suggesting that the basal state of the neural ectoderm is anterior and that additional factors are required to generate the more posterior parts of the nervous system. Our results agree with these studies and provide strong evidence that retinoid signaling is required for the expression of posterior neural markers and the correct spatial restriction of anterior markers.

Basic fibroblast growth factor (bFGF) has recently been shown to posteriorize anterior neuroectoderm, in vitro, thus suggesting a role in neural induction and patterning (Cox and Hemmati-Brivanlou, 1995; Kengaku and Okamoto, 1995; Lamb and Harland, 1995; reviewed by Doniach, 1995). Recent experiments utilizing transgenic embryos expressing the dominant negative FGF receptor, XFD, show that blocking endogenous FGF receptor signaling results in strong inhibition of posterior mesoderm but in only limited effects on A/P patterning of the nervous system (Kroll and Amaya, 1996; Ponwall et al., 1996). Our results show that blocking retinoid signaling alone is sufficient to ablate posterior markers. Assuming that endogenous FGFs and receptors are present in our microinjected embryos, this suggests either that signaling through the FGF receptor is not completely sufficient for neural posteriorization or that components of the FGF signaling pathway are retinoid dependent. Consistent with our results, Maden et al. (1996) showed that vitamin A deficient quail embryos have A/P neural tube defects, including loss of the hindbrain marker, Krox-20. It is clear from our results and theirs that retinoids cannot be the only factor required for posteriorization. Since embryos injected with the dominant negative RARα do have tails, although not posterior markers, there must be additional factors that participate in restricting anterior, and promoting posterior development. Such factors could involve signaling through other RARs and RXRs, as yet unidentified FGF-like receptors or members of the vertebrate hedgehog or Wnt gene families. Taken together, these results suggest that retinoid signaling is critical for correct A/P neural patterning. Whether retinoids act as gradient morphogens, as has been postulated for the posteriorizing signal (Gilbert and Saxen, 1993), or instead as discrete local signals (Bryant and Gardiner, 1992) remains to be investigated.

In summary, our results demonstrate that constitutively active receptors reduce anterior neural tissue while dominant negative receptors expand anterior neural structures. Furthermore, retinoid signaling is required for the expression of posterior markers but is not required for the establishment of the anterior genetic program. We infer that xRAR activation promotes or activates the posterior genetic program (posterior hindbrain and spinal cord) while functioning to restrict the expression of forebrain genes to the anterior of the embryo. These data show that retinoid signaling is required to correctly position regional marker genes along the A/P axis. In addition to their role in neural patterning, retinoids and their receptors are required for neuronal differentiation. Future investigation of the role of retinoids and their receptors in axial patterning and their interaction with other signaling pathways will be important for understanding how multiple signals are integrated during regional specification along the A/P axis.

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