Xrx1 controls proliferation and neurogenesis in *Xenopus* anterior neural plate

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Summary

In *Xenopus* neuroectoderm, posterior cells start differentiating at the end of gastrulation, while anterior cells display an extended proliferative period and undergo neurogenesis only at tailbud stage. Recent studies have identified several important components of the molecular pathways controlling posterior neurogenesis, but little is known about those controlling the timing and positioning of anterior neurogenesis. We investigate the role of *Xrx1*, a homeobox gene required for eye and anterior brain development, in the control of proliferation and neurogenesis of the anterior neural plate. *Xrx1* is expressed in the entire proliferative region of the anterior neural plate delimited by cells expressing the neuronal determination gene *X-ngnr-1*, the neurogenic gene *X-Delta-1*, and the cell cycle inhibitor *p27Xic1*. Positive and negative signals position *Xrx1* expression to this region. *Xrx1* is activated by *chordin* and Hedgehog gene signaling, which induce anterior and proliferative fate, and is repressed by the differentiation-promoting activity of neurogenin and retinoic acid. *Xrx1* is required for anterior neural plate proliferation and, when overexpressed, induces proliferation, inhibits *X-ngnr-1*, *X-Delta-1* and *N-tubulin* and counteracts *X-ngnr-1*- and retinoic acid-mediated differentiation. We find that *Xrx1* does not act by increasing lateral inhibition but by inducing the antineurogenic transcriptional repressors *Xhairy2* and *Zic2*, and by repressing *p27Xic1*. The effects of *Xrx1* on proliferation, neurogenesis and gene expression are restricted to the most rostral region of the embryo, implicating this gene as an anterior regulator of neurogenesis.

Key words: *Xrx1*, *Xhairy2*, *Zic2*, *p27Xic1*, *XBF-1*, *Xenopus laevis*, Proliferation, Neurogenesis, Retinoic acid

Introduction

Genetic studies in *Drosophila* have been instrumental in identifying molecular pathways involved in the control of neurogenesis. This process is initially regulated by positional information provided by prepatterning genes that control the site-specific expression of proneural genes (Gomez-Skarmeta et al., 1996). The role of proneural genes, which code for transcription factors of the basic helix-loop-helix (bHLH) class, is to define clusters of cells competent to originate neuronal precursors (Chitnis, 1999). Within each cluster, a single cell is selected to become a neuroblast by the expression of neurogenic genes. This is accomplished by lateral inhibition, a process mediated by the membrane-bound ligand Delta and the Notch receptor. One cell in the cluster becomes committed to a neuronal fate by expressing higher levels of Delta, which in turn activates the Notch receptor in adjacent cells, forcing them to remain uncommitted. The search for vertebrate homologues of proneural and neurogenic genes has led to the discovery that key regulators of neurogenesis are evolutionary conserved (Chitnis, 1999). In particular, many studies on vertebrate neural induction and neurogenesis have been performed in the amphibian *Xenopus laevis* because of the experimental accessibility of its embryos and the small number of early differentiating primary neurons. In this species, it was shown that the dorsal mesodermal region called Spemann's organizer (Spemann, 1938) neuralizes the dorsal ectoderm by secreting noggin, chordin and follistatin which antagonize BMP4, an epidermalizing signal (Sasai and De Robertis, 1997). Following neural induction, the spatial distribution of neuronal precursors within the posterior neural plate is controlled by prepattern genes of the Xiro, Gli and Zic families. In particular, Zicr1, Xzic3 and Gli proteins induce neurogenesis, while Zic2, Xiro1, Xiro2 and Xiro3, acting as anti-neurogenic transcription factors, restrict the expression domains of proneural genes (Nakata et al., 1997; Mizuseki et al., 1998; Brewster et al., 1998; de la Calle-Mustienes et al., 2002). Regulators of these transcription factors include the Hedgehog genes, which promote proliferation by repressing *Gli3* and activating *Zic2*, and retinoic acid (RA), a posteriorizing morphogen that induces neuronal differentiation by inhibiting the expression of Hedgehog genes (Franco et al., 1999). As early as the end of gastrulation, the first sites of

neurogenesis within the neural plate are marked by the expression of *X-ngnr-1*, which encodes an atonal type bHLH protein, followed by the activation of *X-Delta-1* and finally of *N-tubulin*, a marker of differentiated neurons. The expression of these genes is restricted to three longitudinal rows on either side of the dorsal midline, where individual cells are selected for differentiation through the action of Delta/Notch (Chitnis, 1999).

Although the molecular mechanisms underlying the control of neurogenesis in the posterior nervous system are beginning to be unraveled, less is known about factors controlling neuronal differentiation in the anterior neural plate. Lineage tracing and pulse-labeling experiments (Hartenstein, 1989; Eagleson et al., 1995), as well as analysis of neuronal differentiation markers (Hartenstein, 1993; Papalopulu and Kintner, 1996), have shown that anterior neural plate cells undergo neuronal differentiation significantly later than cells of the posterior neural plate. An as yet unanswered question is what are the factors controlling this phenomenon and how they are related to regulators of posterior vertebrate neurogenesis. So far, only a small group of transcription factors expressed in the anterior neural plate, including *XBF-1*, *Xanf-1*, *Xsix3* and *Xoptx2*, have been shown to play a role in delaying neuronal differentiation and/or promoting proliferation (Bourgouignon et al., 1998; Ermakova et al., 1999; Zuber et al., 1999; Bernier et al., 2000; Hardcastle and Papalopulu, 2000). However, because the spatiotemporal expression of these genes does not coincide with the entire proliferative region of the anterior neural plate, additional genes are likely to be involved. In this work, we propose that *Xrx1*, a homeobox gene required for eye and anterior brain development, is one such factor. We report that *Xrx1* is expressed in the entire proliferative anterior neural plate surrounded by cells expressing *X-ngnr-1*, *X-Delta-1* and *p27Xic1*, a cell cycle inhibitor. *Xrx1* microinjection inhibits *X-ngnr-1*, *X-Delta-1* and *N-tubulin* expression, and counteracts RA- and *X-ngnr-1*-mediated differentiation, while at the same time activating proliferation. These effects are independent of Notch signaling and are restricted to the most rostral region of the embryo. *Xrx1* exerts its function by activating *Xhairy2* and *Zic2*, the expression of which in the anterior neural plate overlaps with that of *Xrx1*, and by repressing *p27Xic1*. Accordingly, loss-of-function experiments show that *Xrx1* is required for the normal proliferation of the anterior neural plate. These data indicate that *Xrx1* possesses the appropriate activities and spatiotemporal expression pattern to be one of the factors responsible for the maintenance of anterior neuronal precursors in a proliferative state.

Materials and methods

Embryo manipulations and whole-mount in situ hybridization

Xenopus embryos were generated and staged as described (Nieuwkoop and Faber, 1967; Newport and Kirschner, 1982). Whole-mount in situ hybridization on embryos and animal caps was performed essentially as described by Harland (Harland, 1991). Histological examination was performed according to Casarosa et al. (Casarosa et al., 1997). For HUA treatment, stage 10 devitellinised embryos were added to a 20 mM hydroxyurea, 150 μM aphidicolin in 0.1× MMR solution, as described by Hardcastle and Papalopulu (Hardcastle and Papalopulu, 2000), and kept in this solution until fixation.

Embryo microinjections, animal cap assay, immunostaining, and BrdU incorporation

Capped synthetic RNAs encoding for *Xrx1* (20-100 pg), *X-chh* (1 ng), *X-shh* (1 ng) (Ekker et al., 1995), *X-ngnr-1* (40 pg) (Ma et al., 1996), *XRALDH2* (1.5 ng) (Chen et al., 2001), *Notch-ICD* (30 pg-1.8 ng) (Chitnis et al., 1995), *X-Delta-1*^{stu} (500 pg) (Chitnis et al., 1995), *XBF-1* (150 pg) (Bourgouignon et al., 1998) and *Xhairy2* (125 pg) (Davis et al., 2001) were generated by in vitro transcription and co-injected with *lacZ* RNA(100-500 pg) into one blastomere at the two-cell stage or into a dorsal blastomere at the four-cell stage. The optimal concentration of each batch of RNA was identified through injection of various doses followed by analysis of either the phenotype or the expression of specific markers. For animal cap experiments, capped synthetic *chordin* (150 pg per blastomere) (Sasai et al., 1995), *X-ngnr-1* (40 pg per blastomere) and *Xrx1* (360 pg per blastomere) RNAs were injected into both blastomeres at the two-cell stage and animal caps dissected at stage 9. When sibling control embryos reached stage 16 or 17, animal caps were fixed and stored in ethanol at –20°C. For retinoic acid treatment, injected animal caps were incubated in 2×10^{-6} M RA in 0.5×MMR where they were cultured until stage 16. For the experiments shown in Fig. 2I-L, Fig. 3E, Fig. 5P,Q, the total amount of RNA injected, either in the experimental or in the respective control samples, is the same. This was achieved by adjusting the amount of *lacZ* RNA in the control samples. The *Xrx1* antisense morpholino used was: 5′-TCAGGGAAGGGCTGTGCA-GGTGCAT-3′ (Gene Tools LLC). A standard morpholino oligo (Gene Tools LLC) was injected as control. Immunostaining with anti-phosphorylated H3 antibody was performed as described by Saka and Smith (Saka and Smith, 2001). BrdU incorporation was performed essentially as described by Hardcastle and Papalopulu (Hardcastle and Papalopulu, 2000).

Results

Factors localizing *Xrx1* expression in the anterior neural plate

Xrx1 is a homeobox gene initially expressed in the anterior neural plate in territories fated to give rise to the retina, diencephalon and part of the telencephalon. Its overexpression induces overgrowing of the neural retina, pigmented epithelium and anterior neural tube, while inhibition of its function leads to a strong reduction or absence of the eye and anterior brain (Casarosa et al., 1997; Mathers et al., 1997; Andreazzoli et al., 1999). As cells of the anterior neural plate are characterized by prolonged proliferation and delayed neuronal differentiation, we decided to analyze whether *Xrx1* is involved in the control of these activities. To better define *Xrx1* expression in the context of early neurogenesis, we compared the expression domain of *Xrx1* with that of early neuronal differentiation markers. Double in situ hybridization experiments showed that at early neurula *Xrx1* is expressed in a territory that is precisely circumscribed by the expression of the neurogenic gene *X-Delta-1* and abuts on the anterior expression domains of the neuronal determination gene *X-ngnr-1* (Fig. 1A,B). Histological sections showed that within this area *Xrx1* is expressed exclusively in the deep sensorial layer of the neuroectoderm where both primary and secondary neurons will form (Hartenstein, 1989) (Fig. 1C). Regions marked by *X-Delta-1* and *X-ngnr-1* expression coincide with prospective sites of neuronal differentiation. In particular, the most anterior semicircular stripes of *X-Delta-1* and *X-ngnr-1* correspond to the presumptive olfactory placodes, part of the telencephalon and laterally to the epiphysis, while the more

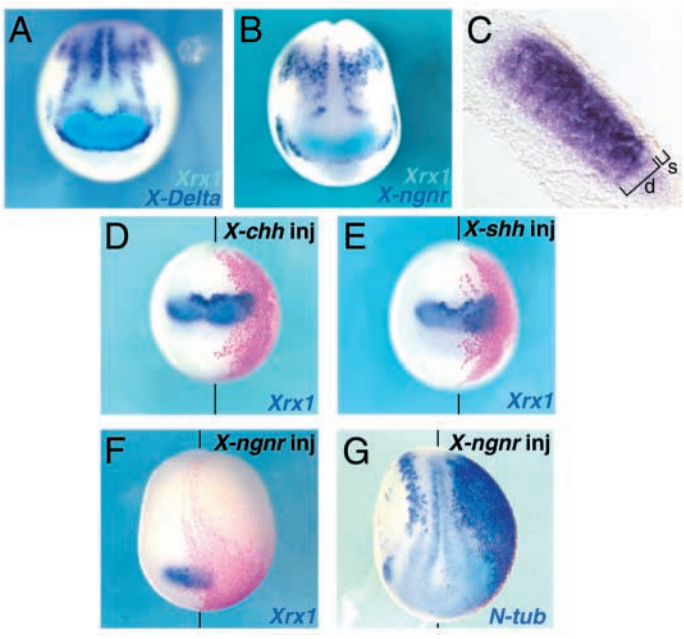

Fig. 1. *Xrx1* expression in the proliferative region of the anterior neural plate is controlled by Hedgehog and *neurogenin* signaling. (A,B) Expression of *Xrx1* (light blue) in relation to the expression of (A) *X-Delta-1* (purple) and (B) *X-ngnr-1* (purple) in stage 13 embryos; frontodorsal views. (C) Sagittal section of a stage 13 embryo showing *Xrx1* expression in the deep sensorial layer of the neuroectoderm. (D,E) Stage 14 embryos injected with *X-chh* (D) and *X-shh* (E) showing ectopic expression of *Xrx1* (blue); frontal views, dorsal towards the top. (F,G) Embryos injected with *X-ngnr-1* displaying repression of *Xrx1* (blue, F, stage 13) and ectopic expression of *N-tubulin* (blue, G, stage 16); frontodorsal views. The injected side of the embryos (to the right of vertical bars representing the midline) is indicated (inj). Red staining represents expression of co-injected *lacZ* lineage tracer. d, deep neuroectodermal layer; s, superficial neuroectodermal layer.

posterior *X-Delta-1* medial stripe coincides with the ventral midbrain (Eagleson and Harris, 1990; Bourguignon et al., 1998). Although expression of several other genes partially overlaps with that of *Xrx1*, to our knowledge *Xrx1* is the only gene described so far whose expression completely fills the anterior gap of *X-Delta-1* expression, thus corresponding exactly to the proliferative region of the anterior neural plate.

We next investigated which factors localize *Xrx1* expression to the proliferative region of the anterior neural plate. Because the Hedgehog genes were shown to induce proliferation and delay differentiation in the early neural plate (Franco et al., 1999), we decided to test if they affect *Xrx1* expression. We found that both *sonic hedgehog* (*X-shh*) and *cephalic hedgehog* (*X-chh*) are able to activate *Xrx1* at early neurula stage (*X-shh* 68%, *n*=44; *X-chh* 56%, *n*=36; Fig. 1D,E). *Xrx1* is ectopically activated by Hedgehog signaling only in an area that surrounds the endogenous *Xrx1* expression domain, despite of the broader distribution of the injected RNA. Because of the lack of *Xrx1* expression in *X-Delta-1* and *X-ngnr-1* positive regions, we looked if proneural gene expression plays a role in restricting *Xrx1* expression. Overexpression of *X-ngnr-1*, which efficiently induces ectopic *N-tubulin* expression (100%, *n*=31; Fig. 1G), strongly represses *Xrx1* expression (100%, *n*=32; Fig. 1F). These data indicate that *Xrx1* expression is not compatible with neuronal differentiation and that the anterior expression of proneural genes like *X-ngnr-1* defines the perimeter of the *Xrx1* expression domain.

Xrx1 inhibits neuronal differentiation

The coincidence of *Xrx1* expression with the proliferating area of the anterior neural plate, where neuronal markers are not expressed, led us to think that *Xrx1* might be part of the system preventing precocious neurogenesis in this area. To test this hypothesis, we analyzed the expression of neuronal differentiation markers in *Xrx1*-injected embryos during early neurulation. We observed that *X-ngnr-1*, *X-Delta-1* and *N-tubulin* are all repressed in the anterior region by *Xrx1* overexpression (*X-ngnr-1*, 91%, *n*=58; *X-Delta-1*, 90%, *n*=31; *N-tubulin*, 97%, *n*=92; Fig. 2A-D), while the repressive effects are weak in the posterior expression domains of these markers (Fig. 2A-C,E). *Sox2*, a general neural marker, was not affected at this stage (0%, *n*=90; Fig. 2F), indicating that *Xrx1* acts on neuronal differentiation but not on neural induction. As a positive control, *Xrx1* ectopically activates *XBF-1* in the lateral border of the neural plate (58%, *n*=24; Fig. 2G), as previously described (Andreazzoli et al., 1999). *Xrx1* effects on neurogenesis are distinct from those observed upon *XBF-1* overexpression (Bourguignon et al., 1998). In fact, injection of *XBF-1* at doses that cause suppression of endogenous *N-tubulin* also leads to ectopic activation of *N-tubulin* along the boundary of the injected area in the posterior neural plate (94%, *n*=36; Fig. 2H). In a complementary approach, we tested if *Xrx1* has the ability to inhibit ectopic neurogenesis induced by *X-ngnr-1* overexpression. Injection of *X-ngnr-1* induced a massive expression of *N-tubulin*, the in situ signal of which covered the β-gal staining (100%, *n*=41; Fig. 2I, also compare the injected versus uninjected side in Fig. 1G). At variance, co-injection of *X-ngnr-1* and *Xrx1* resulted in a considerable attenuation of *N-tubulin* activation (95% with reduced ectopic expression, *n*=45; Fig. 2K). These data were confirmed by animal cap experiments where *X-ngnr-1* ability of inducing *N-tubulin* (Ma et al., 1996) was inhibited by *Xrx1* (*X-ngnr-1* + *lacZ* 100% positive, *n*=58; *X-ngnr-1* + *Xrx1* 96% negative, 4% weakly positive, *n*=62; Fig. 2J,L).

Xrx1 counteracts RA differentiating signals

Retinoic acid has been shown to control the timing of neuronal differentiation being able to accelerate neurogenesis in anterior neural cells (Papalopulu and Kintner, 1996; Sharpe and Goldstone, 2000). Although RA is thought to function mainly in the posterior neural plate and mesoderm during early development (Chen et al., 1994), it has been shown recently that *XRALDH2*, one of enzymes involved in RA synthesis, is expressed also in an anterior site (Chen et al., 2001). A double in situ hybridization revealed that *Xrx1* expression adjoins, but does not overlap, the *XRALDH2* anterior expression domain (Fig. 3A). To determine the causes of this spatial relationship between *Xrx1* and *XRALDH2*, we looked at the effect that the overexpression of each of these genes exerts on the other. We found that *Xrx1* and *XRALDH2* exhibit mutually repressive activities (*XRALDH2*-injected embryos: 75% with reduced *Xrx1* expression, *n*=24; Fig. 3B; *Xrx1*-injected embryos: 83% with reduced *XRALDH2* expression, *n*=24; Fig. 3C), which could explain the generation of adjacent, non-overlapping

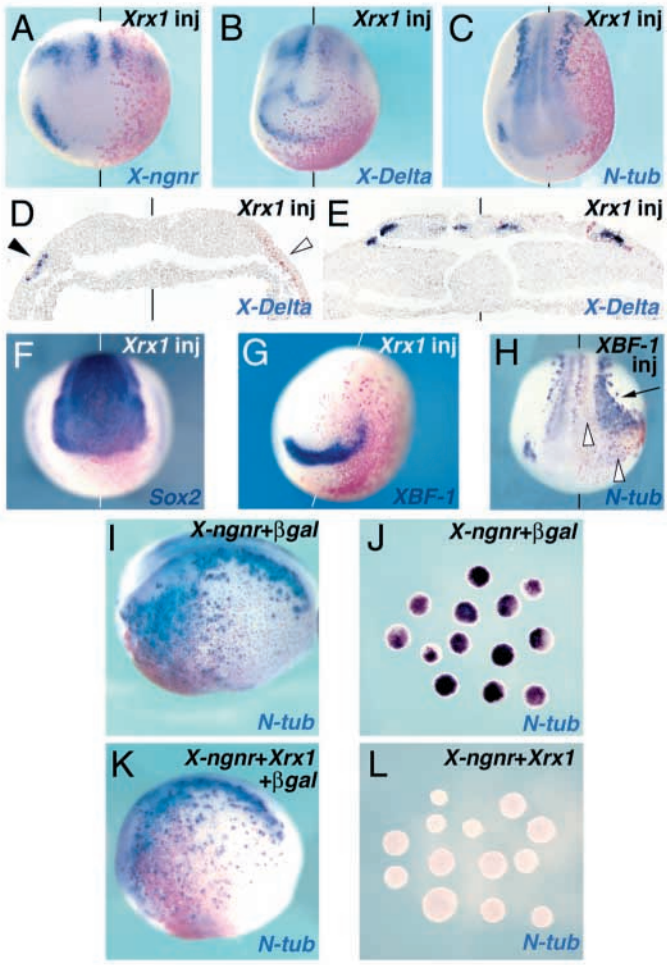

Fig. 2. *Xrx1* inhibits neurogenesis. The effects of *Xrx1* overexpression on (A) *X-ngnr-1* (stage 12), (B,D,E) *X-Delta-1* (stage 13), (C) *N-tubulin* (stage 16), (F) *Sox2* (stage 13) and (G) *XBF-1* (stage 13) are shown. (D,E) Transverse sections at the level of the anterior (D) and posterior (E) neural plate. The black arrowhead indicates the anterior expression domain of *X-Delta-1*, while the white arrowhead indicates the repression of the corresponding domain in the injected side. (H) Stage 16 embryo injected with 150 pg of *XBF-1* RNA showing suppression of endogenous *N-tubulin* (white arrowheads) as well as ectopic *N-tubulin* activation at the border of the injected area (arrow). A-C,F,G,H Frontodorsal views. (I,K) Stage 16 embryos co-injected with either *X-ngnr-1* and *lacZ* (I) or *X-ngnr-1*, *Xrx1* and *lacZ* (K); lateral views, anterior towards the left. (J,L) Animal caps co-injected with either *X-ngnr-1* and *lacZ* (J) or *X-ngnr-1* and *Xrx1* (L) analyzed at stage 16. Both in whole embryos and in animal caps *Xrx1* inhibits *N-tubulin* expression induced by *X-ngnr-1*. Red staining represents expression of co-injected *lacZ* lineage tracer.

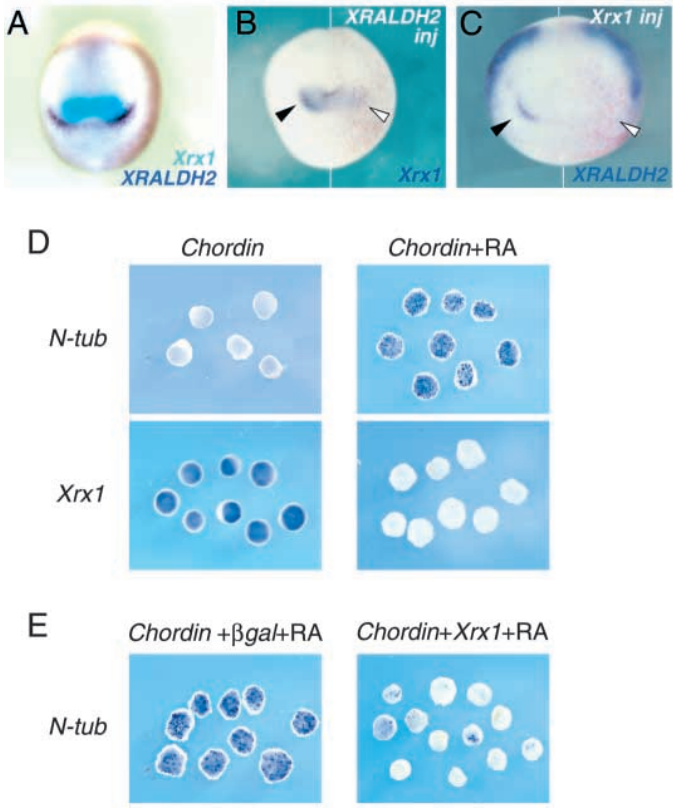

Fig. 3. *Xrx1* counteracts retinoic acid-mediated neuronal differentiation. (A) Comparison of the anterior expression of *XRALDH2* (purple) with that of *Xrx1* (light blue) in a stage 13 embryo. (B) Stage 13 embryo injected with *XRALDH2* showing reduction of *Xrx1* expression. (C) Stage 13 embryo injected with *Xrx1* showing repression of the anterior *XRALDH2* expression domain. Black arrowheads indicate the expression domains of *Xrx1* (B) and *XRALDH2* (C) in the uninjected side of the embryos. White arrowheads indicate the repression of the corresponding domains in the injected side. (A-C) Frontal views, dorsal towards the top. Red staining represents expression of co-injected *lacZ* lineage tracer. (D) Animal caps injected with *chordin* and analyzed at stage 16 express *Xrx1* but not *N-tubulin*. If *chordin*-injected caps are treated with RA at stage 9 and analyzed at stage 16, *Xrx1* expression is suppressed and *N-tubulin* expression is induced. (E) Stage 16 animal caps co-injected with either *chordin* and *lacZ* (control) or *chordin* and *Xrx1*, and treated with RA at stage 9. *Xrx1* strongly inhibits the induction of *N-tubulin* expression.

expression domains. To analyze if *Xrx1* could also counteract the effects of RA on neuronal differentiation, we took advantage of an animal cap system that recapitulates anterior neurogenesis. Papalopulu and Kintner (Papalopulu and Kintner, 1996) showed that *noggin*-injected animal caps cultured until neurula stage (stage 16) express NCAM but not *N-tubulin*. Initiation of *N-tubulin* expression is observed only when these explants are cultured until tailbud stage (stage 27); addition of RA accelerates this process leading to activation of *N-tubulin* by stage 16. We used *chordin* as a BMP antagonist in animal caps, and found that at stage 16 *chordin* alone does not induce *N-tubulin* (0% positive, *n*=26), while RA treatment of *chordin*-injected animal caps robustly activates a punctate expression of *N-tubulin* (97% positive, *n*=33; Fig. 3D). Interestingly, *Xrx1* expression, which is strongly induced in *chordin*-injected animal caps (96% positive, *n*=25), is completely abolished by RA treatment (0% positive, *n*=26; Fig. 3D). This observation again inversely correlates *Xrx1* expression and neurogenesis. To test whether the repression of *Xrx1* was required to activate neurogenesis we treated with RA animal caps that had been co-injected with *chordin* and *Xrx1* RNA. Co-injection of *Xrx1*, but not of *lacZ*, effectively inhibited *N-tubulin* expression in RA-treated caps (*Chordin* + *lacZ* 100% positive, *n*=25; *Chordin* + *Xrx1* 80% negative, 20%

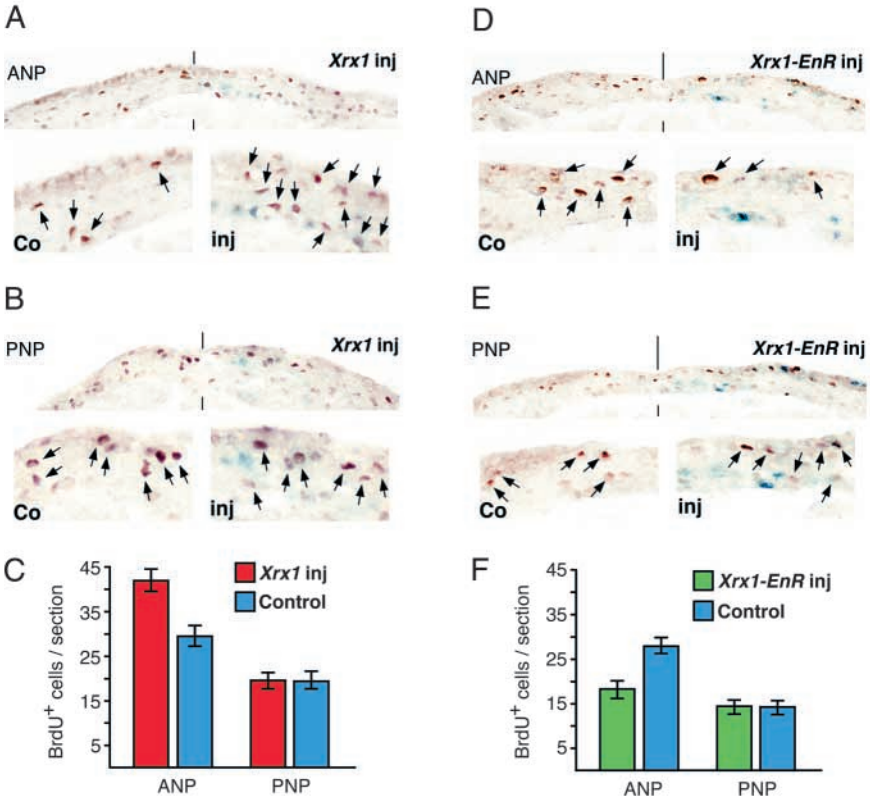

Fig. 4. *Xrx1* supports proliferation in the anterior neural plate. (A,B,D,E) Transverse sections of stage 13 embryos injected with either *Xrx1* (A,B) or *Xrx1-EnR* (D,E) and processed for BrdU incorporation (brown nuclear staining). Sections at the level of anterior neural plate (A,D) and posterior neural plate (B,E) are shown. Turquoise staining represents expression of co-injected *lacZ*. Areas with the highest level of β-gal in the injected side (inj) and the corresponding regions in the control side (Co) are shown at high magnification at the bottom of each panel. Arrows indicate BrdU-positive cells. (C,F) The average number of BrdU-positive cells per section in either the control side (blue) or the injected side of *Xrx1* (C, red) and *Xrx1-EnR* (F, green) injected embryos. Error bars indicate s.e.m. ANP, anterior neural plate; PNP, posterior neural plate.

weakly positive, *n*=30; Fig. 3E). Thus, *Xrx1* appears to counteract RA-mediated neuronal differentiation through a dual action: upstream of RA production by repressing the expression of *XRALDH2*, and downstream of RA or acting on a parallel pathway, as shown by its ability to impede RA-mediated differentiation in *chordin*-injected caps.

Xrx1 controls proliferation at early neurula stage in a region-specific manner

Cells of the anterior neural plate are not only characterized by delayed differentiation but also display a protracted proliferating state. Therefore, we asked whether *Xrx1* plays a role in the control of proliferation at early neurula stage. To achieve this, embryos injected with either *Xrx1* or *Xrx1-EnR*, a dominant-negative construct (Andreazzoli et al., 1999) were tested for BrdU incorporation. In these experiments the number of BrdU-positive cells in the injected side was compared with that of the uninjected control side taking also into account their anteroposterior distribution. We found that the anterior neural plate of *Xrx1*-injected embryos displayed a 44% increase of BrdU-positive cells in the injected side compared with the control side (an average of 42.2 positive cells per section in the injected side, *n*=1099, versus 29.2 cells per section in the control side, *n*=760; $P<0.001$; Fig. 4A,C). On the contrary, no significant difference was detected in the posterior neural plate of *Xrx1*-injected embryos (an average of 19.6 positive cells per section in the injected side, *n*=413, versus 19.3 cells per section in the control side, *n*=406; Fig. 4B,C). However, the anterior neural plate of *Xrx1-EnR*-injected embryos shows a 33% decrease of BrdU-positive cells in the injected side compared with the control side (an average of 18 positive cells per section in the injected side, *n*=519, versus 26.8 cells per section in the control side, *n*=773; $P<0.001$; Fig. 4D,F). In addition, no significant difference was observed in the posterior neural plate (an average of 14.3 positive cells per section in the injected side, *n*=253, versus 13.9 cells per section in the control side, *n*=235; Fig. 4E,F). Altogether, these results suggest that *Xrx1* is involved in controlling cell proliferation specifically in the anterior region of the neural plate.

Factors mediating *Xrx1* activities

To gain insight into how *Xrx1* may exert its effects, we analyzed the expression of potential *Xrx1* target genes in injected embryos. The criteria that we used to select the genes tested are the following: (1) the gene has to be expressed in an area where *Xrx1* is also expressed, or in a surrounding region affected by *Xrx1* overexpression; (2) it must play a role in controlling cell differentiation and/or proliferation. *Zic2*, a gene encoding a zinc-finger transcription factor, has been shown to have an antineurogenic function (Brewster et al., 1998). Besides being expressed in the posterior neural plate in stripes that alternate with the primary neurons, *Zic2* is also expressed in the anterior neural plate. Comparing the expression profiles of *Xrx1* and *Zic2*, we found that they mostly overlap in the presumptive forebrain, although *Zic2* RNA extends slightly more to the anterior and *Xrx1* expression more to the posterior (Fig. 5A,B). Overexpression of *Xrx1* leads to an expansion of *Zic2* expression, which extends along the mediolateral axis (95%, *n*=62; Fig. 5D,E), while no ectopic expression is observed in the majority of cases in the posterior neural plate. *Xhairy1* and *Xhairy2* are homologues of the *Drosophila hairy* gene that are known to act as transcriptional repressors and to inhibit neuronal differentiation (Dawson et al., 1995; Koyano-Nakagawa et al., 2000). Both genes display diffuse expression in the anterior neural plate that overlaps with *Xrx1* expression (Fig. 5C,G). Moreover, *Xhairy2*, but not *Xhairy1*, shows a stronger stripe of expression that coincides with the anterior and lateral borders of *Xrx1* expression. Injection of *Xrx1* induces ectopic expression of *Xhairy2*, which, as in the case of *Zic2*, does not extend posteriorly (72%, *n*=50; Fig. 5F). In addition, overexpression of *Xrx1* also leads to a repression of the

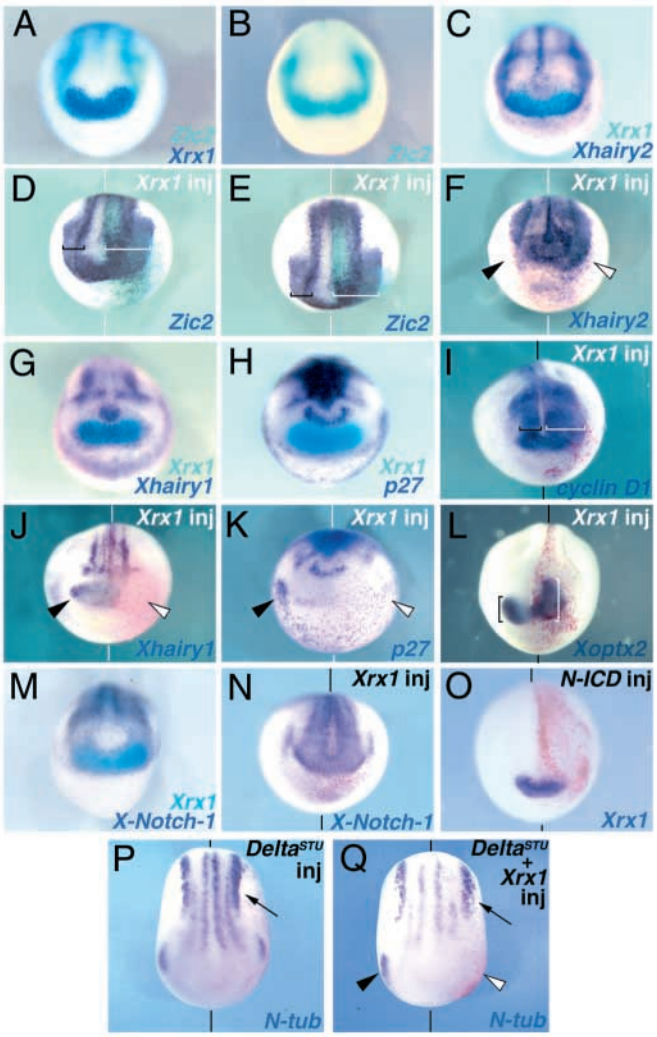

Fig. 5. *Xrx1* regulates the expression of genes that control cell proliferation and differentiation, and does not work through the Notch-Delta pathway at early neurula. (A-C,G,H,M) Comparison of the expression of *Xrx1* to that of *Zic2* (A,B), *Xhairy2* (C), *Xhairy1* (G), *p27Xic1* (H) and *X-Notch-1* (M) in stage 13 embryos. (D-F,I-L,N) *Xrx1*-injected embryos analyzed at stage 14 (D-F,J,K,N) or stage 18 (I,L). The probes used and the respective staining are indicated, color-coded, on the bottom of each panel. (A-D,F-O) Frontal views, dorsal towards the top; (E,P,Q) dorsoanterior views. The injected side of the embryos (to the right of vertical bars representing the midline) is indicated (inj). Red staining in F,I,J,K,L,N-Q and turquoise staining in D,E represent expression of co-injected *lacZ* lineage tracer. (F,J,K) Black arrowheads indicate the lateroventral expression domain of *Xhairy2* (F), *Xhairy1* (J) and *p27Xic1* (K) in the uninjected control side of the embryos. White arrowheads indicate the corresponding expression domain in the injected side, which is expanded in the case of *Xhairy2* (F) and repressed in the case of *Xhairy1* (J) and *p27Xic1* (K). Black brackets indicate the anterior expression domains of *Zic2* (D,E), *cyclinD1* (I) and *Xoptx2* (L) in the control uninjected side; white brackets indicate the corresponding enlarged domains in the injected side. (O) Stage 14 embryo injected with *Notch-ICD* showing no significant change in *Xrx1* expression. (P,Q) Stage 16 embryos injected with *X-Delta-1*^{stu} and *lacZ* (P) or co-injected with *Xrx1*, *X-Delta-1*^{stu} and *lacZ* (Q). *Xrx1* represses *N-tubulin* expression in the trigeminal ganglion but does not affect *N-tubulin* posterior expansion. Arrows indicate the increase in density of *N-tubulin*-positive cells within the posterior neurogenic stripes caused by the block of lateral inhibition. The black arrowhead indicates *N-tubulin* expression in the trigeminal ganglion of the uninjected side; the white arrowhead indicates the absence of this expression domain in the injected side.

anterior domain of *Xhairy1* (90%, *n*=40; Fig. 5J). As *Xhairy1* and *Xhairy2* are known to repress each other, and because there is evidence that *Xrx1* might work as a transcriptional activator (Andreazzoli et al., 1999), it is possible that *Xhairy2* is first activated by *Xrx1* and that this excess of *Xhairy2* could be responsible for the downregulation of *Xhairy1*. *Xoptx2*, a homeobox gene of the Six homeobox family, has been shown to play a role in controlling the proliferation of the retina (Zuber et al., 1999). Analysis of *Xoptx2* expression in *Xrx1*-injected embryos showed that, although at stage 14 the expression of this gene does not appear to be affected (89% normal expression, 11% slightly reduced expression, *n*=67; not shown), a remarkable expansion is observed at stage 18 (75%, *n*=56; Fig. 5L). This response is similar to the one previously described for the related gene *Xsix3* (Andreazzoli et al., 1999). *p27Xic1*, an inhibitor of cyclin/cdk required for primary neurogenesis (Su et al., 1995; Carruthers et al., 2003; Vernon et al., 2003), is expressed anteriorly in two semicircles very similar to those characterized by *X-Delta-1* expression. We observed that *Xrx1* expression is complementary to that of *p27Xic1*, being delimited dorsally and ventrally by the two *p27Xic1* expression domains (Fig. 5H). Overexpression of *Xrx1* suppresses *p27Xic1* expression, particularly in its most anterior domains (96%, *n*=83; Fig. 5K). Finally, we looked at *cyclin D1*, which has a strong expression site in the eye field (http://www.xenbase.org/) (Vernon and Philpott, 2003) and has been implicated in mouse eye development (Fantl et al., 1995). Although *Xrx1*-injected embryos did not show any difference in *cyclin D1* expression at early neurula stage (stage 13, 100% normal expression, *n*=46; not shown), a notable expansion was observed at late neurula stage (stage 18, 83%, *n*=24; Fig. 5I). Thus, *Zic2* and *Xhairy2* are activated and *p27Xic1* is repressed by *Xrx1* at early neurula stage, while *Xoptx2* and *cyclin D1* are activated only at a later stage under the influence of *Xrx1*.

Xrx1 function is not mediated by lateral inhibition during early neurulation

X-Notch-1 is expressed in the anterior neural plate in the region occupied by *Xrx1* (Fig. 5M), and its activity in preventing differentiation of neurons has been described (Chitnis et al., 1995). Moreover, the mouse *Xrx1* homologue (*Rx1*, also called *Rax*) has been shown to activate *Notch* transcription during retinogenesis (Furukawa et al., 2000). For these reasons, we tested whether, during early neurulation, *Xrx1* and *X-Notch-1* affect one another's expression. Analysis of *Xrx1*-injected embryos at various stages during early neurulation failed to show any transcriptional activation of *X-Notch-1* (stage 13, 0%, *n*=54; stage 15, 0%, *n*=33; stage 18, 0%, *n*=38; Fig. 5N). Furthermore, injection of several doses of a constitutively active form of *X-Notch-1* (*Notch-ICD*) (Chitnis et al., 1995) did not show activation of *Xrx1* at early neurula (30 pg 86% normal, 10% slightly reduced expression, 4% slightly expanded expression,

n=30; 500 pg 84% normal, 8% slightly reduced expression, 8% slightly expanded expression, *n*=37; 1.8 ng 85% normal, 11% slightly reduced expression, 4% slightly expanded expression, *n*=27; Fig. 5O). These results suggest that during early neurulation, *Xrx1* is not affected directly by Notch signaling, and that *Xrx1* does not affect *Notch* expression. Finally, to test if the effects of *Xrx1* on neurogenesis are mediated by lateral inhibition, we co-injected *Xrx1* with *X-Delta-1*^{stu}, an antimorphic version of *X-Delta-1* (Chitnis et al., 1995). Even though under these conditions, lateral inhibition was blocked, as shown by an excess of *N-tubulin*-expressing cells in the posterior neural plate (Fig. 5P,Q, arrow), *Xrx1* was still able to repress the trigeminal ganglia expression of *N-tubulin* (75% absent expression, 25% reduced expression, *n*=40; Fig. 5Q).

Xrx1 controls the expression of *Xhairy2*, *Zic2*, *X-ngnr-1* and *p27Xic1* in the absence of cell division

The ectopic expression of *Xhairy2* and *Zic2* as well as the repression of *X-ngnr-1* and *p27Xic1* in *Xrx1*-injected embryos could be triggered independently of cell proliferation or, alternatively, could result from an expansion of the proliferating neuroectoderm. To distinguish between these two possibilities, we asked whether *Xrx1* can affect *Xhairy2*, *Zic2*, *X-ngnr-1* and *p27Xic1* expression in embryos where cell division has been blocked by treatment with hydroxyurea and aphidicolin (HUA) (Harris and Hartenstein, 1991). HUA treatment severely affected anti-phoshoH3 staining, a marker of cells in mitotic prophase, as well as *Xoptx2* ability of expanding *Xrx1* (Zuber et al., 1999) and resulted in smaller embryos with reduced optic vesicles (Fig. 6E-I). Under these conditions, *Xrx1* is still able to expand *Zic2* (96%, *n*=78) and *Xhairy2* (71%, *n*=74) expression and to repress the expression of *X-ngnr-1* (97%, *n*=36) and *p27Xic1* (84%, *n*=44), although not to the same extent as in untreated embryos (Fig. 6A-D). These data suggest that the regulation of *Xhairy2*, *Zic2*, *X-ngnr-1* and *p27Xic1* observed in *Xrx1*-injected embryos does not depend exclusively on proliferation. This observation may be consistent with the finding that *Xrx1* is able to convert competent ectoderm to an anterior neural fate (Kenyon et al., 2001).

Xrx1 activates *Xhairy2*, but not *Zic2*, in isolated ectoderm

As *Xrx1* microinjection expands *Zic2* and *Xhairy2* expression even in HUA-treated embryos, we tested whether *Xrx1* could activate these genes in non-neuralized ectoderm. To achieve this, we analyzed the expression of *Zic2* and *Xhairy2* in *Xrx1*-injected animal caps. At the same time, we also looked at the expression of *XBF-1*, a gene activated by *Xrx1* in the lateral border of the anterior neural plate, *Sox2*, a general neural marker, and *XK81*, an epidermal marker (Fig. 7). Control uninjected caps showed no expression of *Xhairy2*, *Zic2*, *XBF-1* and *Sox2* (0% in all cases; *Xhairy2*, *n*=39; *Zic2*, *n*=32; *XBF-1*, *n*=33; *Sox2*, *n*=41), while they expressed *XK81* (100%, *n*=20). By contrast, *Xrx1*-injected animal caps expressed *Xhairy2* (60% positive, 21% weakly positive, *n*=72) and *XK81* (100%, *n*=32) while showing no activation of *Zic2*, *XBF-1* and a weak activation of *Sox2* (*Zic2*, 0%, *n*=65; *XBF-1*, 0% *n*=37; *Sox2*, 8% weakly positive, *n*=41). Thus, among the genes activated by *Xrx1* in the early neurula embryos, *Xhairy2* appears to be the only one whose induction by *Xrx1* is independent of ectoderm neuralization.

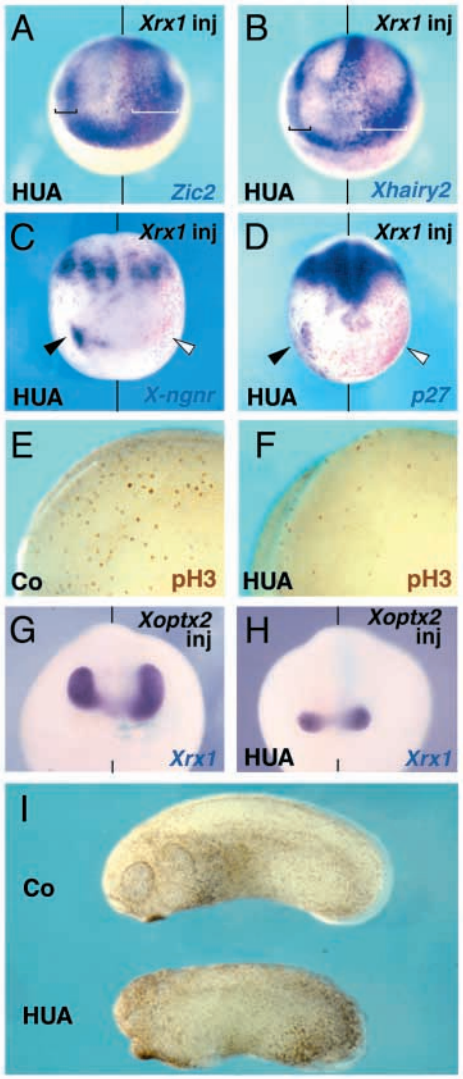

Fig. 6. (A-D) Embryos injected with *Xrx1* were treated with HUA at stage 10.5 and the expression of *Zic2* (A), *Xhairy2* (B), *X-ngnr-1* (C) and *p27Xic1* (D) was analyzed at stage 13. (A,B) Black brackets indicate the anterior expression domains in the uninjected side; white brackets indicate the corresponding enlarged domains in the injected side. (C,D) Black arrowheads indicate anterior expression domains in the uninjected side, white arrowheads indicate the absence of this expression domain in the injected side. (E-I) HUA treatment dramatically reduced anti-phosphoH3 staining (E,F; stage 16) as well as *Xoptx2* ability of expanding *Xrx1* (Zuber et al., 1999) (G,H; stage 18). This treatment also results in a reduction of the optic vesicle size (I; stage 26; Co, control untreated embryo). (A-D,G,H) Frontal views, dorsal towards the top; (E,F,I) lateral views, anterior towards the left. Red staining in A-D and turquoise staining in G,H represent expression of co-injected *lacZ* lineage tracer. The injected side of the embryos (to the right of vertical bars representing the midline) is indicated (inj).

Xrx1 loss-of-function decreases the size of the anterior neural plate and expands *X-ngnr-1* expression

To further analyze the requirement of *Xrx1* function in controlling the expression of genes involved in anterior proliferation and neurogenesis, we used, as an alternative to the

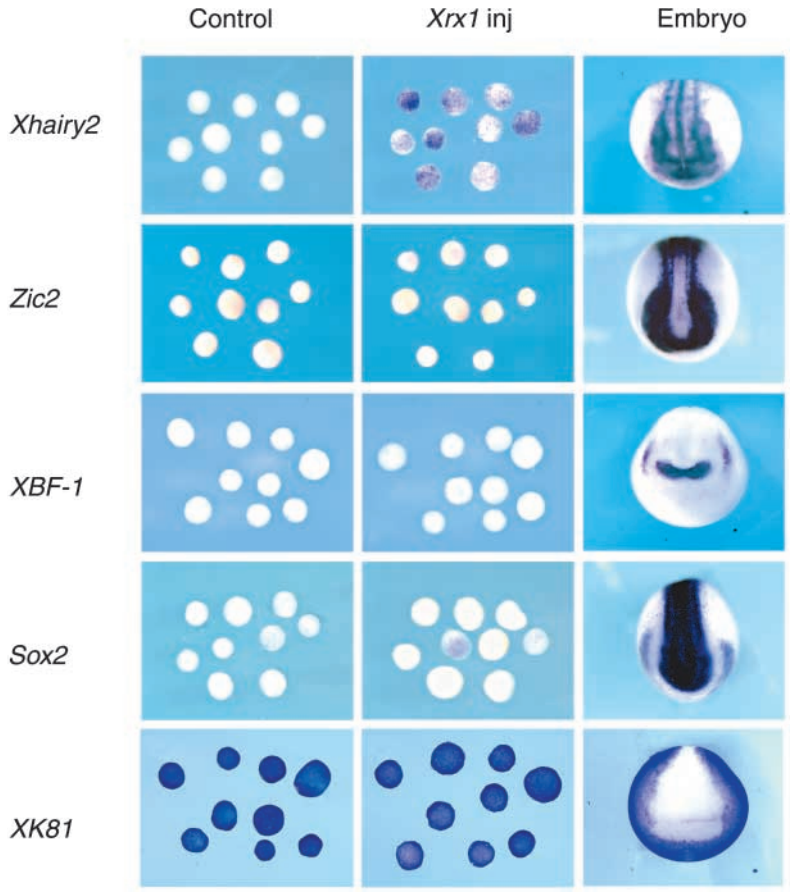

Fig. 7. *Xrx1* induces *Xhairy2* but does not affect several other markers in animal caps. *Xrx1* injected animal caps were dissected at stage 9, cultured to stage 17 and analyzed for the expression of the indicated genes. The column on the right (Embryo) shows the expression of the indicated genes in control embryos.

Xrx1-EnR construct, an antisense morpholino approach (Heasman et al., 2000). Injection of 10 ng of a morpholino oligo directed against *Xrx1* (*MoXrx1*) into dorsoanimal blastomeres at the eight-cell stage generates embryos displaying severe reduction of eyes and anterior head (97%, *n*=102; Fig. 8A). This phenotype, which is very similar to the one obtained after *Xrx1-EnR* injection and to *Rx1*$^{-/-}$ mice (Mathers et al., 1997; Andreazzoli et al.,1999), is completely rescued by co-injection of 80 pg of *Xrx1* RNA (99%, *n*=86; Fig. 8B) and is not observed upon injection of control morpholino (96% normal embryos, *n*=75; Fig. 8C). Analysis of *X-ngnr-1* expression in both *MoXrx1*- and *Xrx1-EnR*-injected embryos showed an anterior expansion of the expression domains of this gene, which tend to fuse medially. This phenotype is first observed at stage 14 (*Xrx1-EnR*: 82% *n*=34; *MoXrx1*: 83%, *n*=79; Fig. 8D-F) and is maintained at stage 18 (*Xrx1-EnR*: 75%, *n*=28; *MoXrx1*: 78%, *n*=38; Fig. 8H-J). The expanded expression domains of *X-ngnr-1* appeared to correspond to the telencephalon rather than the trigeminal ganglia. This was confirmed by the observation that *MoXrx1* did not affect the expression of *FoxD3*, a marker of neural crest cells that contribute also to the trigeminal ganglia (100%, *n*=19; Fig. 8G,K). To analyze simultaneously the effects of *Xrx1* knockdown on neurogenesis and eye-field specification we co-hybridized *MoXrx1*-injected embryos with *X-ngnr-1* and *Xrx1* (Fig. 8L-N). We found that the medial expansion of *X-ngnr-1*correlates with a smaller *Xrx1* expressing area, which in extreme cases was totally abolished, with no overlap of the two markers (85% reduced *Xrx1* expression, Fig. 8M; 15% absent *Xrx1* expression, Fig. 8N, *n*=33). Analogous results were also obtained using *Xsix3* as eye field marker (not shown). A very similar phenotype was observed in HUA-treated embryos (100% reduced *Xrx1* expression, *n*=25; Fig. 8O), suggesting that the effects observed in *Xrx1* loss of function are mainly due to a reduced proliferation of the anterior neural plate. As gain-of-function experiments indicated a potential role of *Xhairy2* downstream of *Xrx1*, we tested whether *Xhairy2* could rescue *MoXrx1* effects. Bilateral injection of *MoXrx1* resulted in reduction, but not complete abolishment, of the expression of *Xhairy2* (98%, *n*=50; Fig. 8P,Q), whereas *Xhairy2* overexpression led to a severe repression of *X-ngnr-1* expression (67%, *n*=36; Fig. 8R). Co-injection of *MoXrx1* and *Xhairy2* resulted in embryos displaying the typical anterior expansion of *X-ngnr-1* observed in embryos injected with *MoXrx1* alone, thus indicating that *Xhairy2* is not able to rescue the *MoXrx1* phenotype (84%, *n*=94; Fig. 8S). Among the genes activated by *Xrx1* overexpression and repressed by *Xrx1* inactivation (Andreazzoli et al., 1999), *XBF-1* is of particular interest, because, like Xrx1, it acts by controlling *p27Xic1* expression (Hardcastle and Papalopulu, 2000). Therefore, we tested whether *XBF-1* could rescue the effects of *MoXrx1* injection. Overexpression of *XBF-1* alone repressed anterior *X-ngnr-1* expression while inducing ectopic *X-ngnr-1* in the posterior neural plate (90%, *n*=30; Fig. 8T,U). When co-injected with *MoXrx1*, *XBF-1* was able to restore a wide *X-ngnr-1*-free area in the anterior neural plate (82%, *n*=70; Fig. 8V). These results suggest that *XBF-1* might work downstream of *Xrx1* and/or that these genes function by controlling common mechanisms.

Discussion

Xrx1 function in early anterior neurogenesis

Comparing the expression domain of *Xrx1* with those of genes involved in promoting neuronal differentiation or blocking cell proliferation, we observed that *Xrx1* expression is complementary to the expression of *X-ngnr-1*, *X-Delta-1*, *XRALDH2* and *p27Xic1*. In particular, *Xrx1* completely fills the area surrounded by the anterior domain of *X-Delta-1*. Other transcription factors known to control neurogenesis in this area include *XBF-1*, *Xoptx2* and *Xanf-1* (Bourgouignon et al., 1998; Ermakova et al., 1999; Zuber et al., 1999). *XBF-1* is expressed only in the most anterior border of the neural plate, adjacent to the rostral expression of *X-Delta-1* and *p27Xic1* (Bourgouignon et al., 1998; Hardcastle and Papalopulu, 2000), where it plays a crucial role in defining the border between proliferative and non-proliferative areas. *Xoptx2* is expressed in the anterior neural plate later than *Xrx1* (Zuber et al., 1999) and *Xanf-1* is expressed in a domain broader than the region

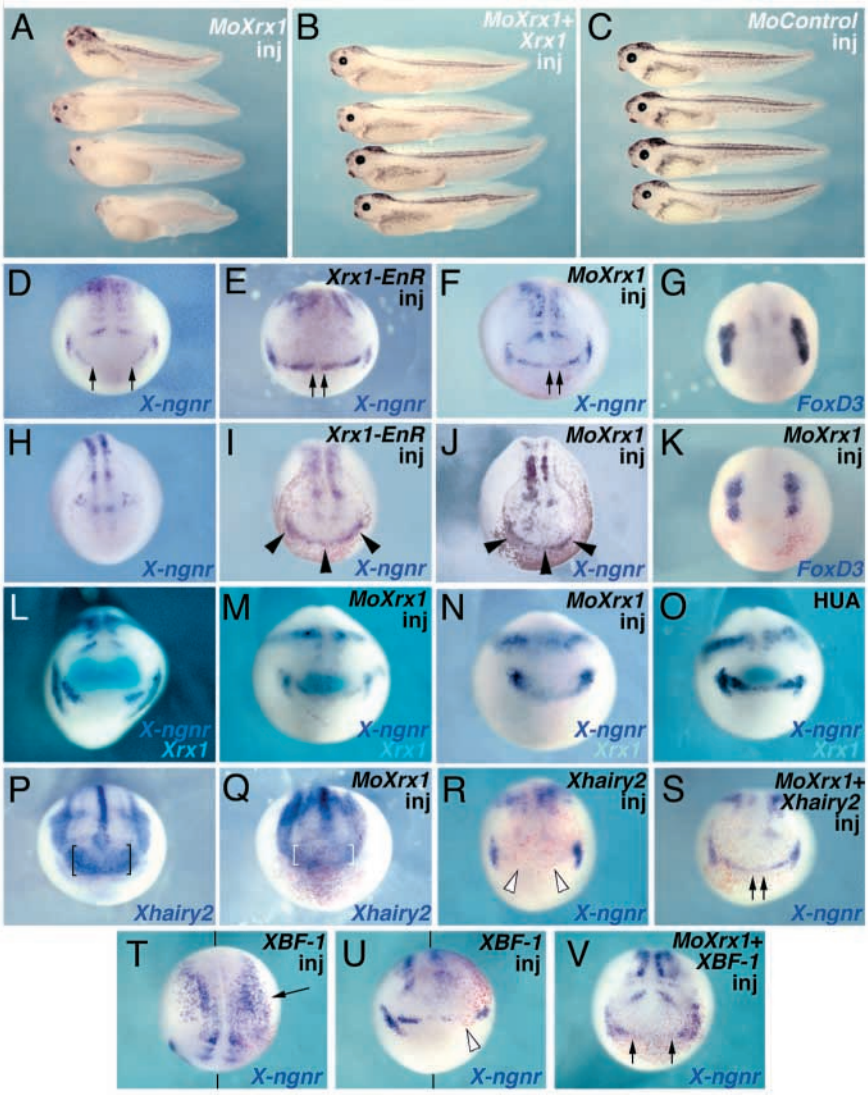

Fig. 8. Effects of *Xrx1* loss of function on genes regulating anterior neurogenesis. (A-C) Phenotypes of stage 41 embryos injected with *MoXrx1* (A), *MoXrx1* and *Xrx1* (B), and control morpholino oligo (C). (F,J,K,M,N,Q) Embryos injected with *MoXrx1* in both dorsoanimal blastomeres at the eight-cell stage and analyzed at stage 14 (F,K,M,N,Q) and stage 18 (J). (D,G,H,L,P) Control uninjected embryos analyzed at stage 14 (D,G,L,P) and stage 18 (H). (E,I) Embryos bilaterally injected with *Xrx1-EnR* analyzed at stage 14 (E) and stage 18 (I). (O) Stage 14 embryo treated with HUA. (R,S,V) Stage 14 embryos bilaterally injected with *Xhairy2* (R), *MoXrx1* and *Xhairy2* (S), and *MoXrx1* and *XBF-1* (V). (T,U) Dorsal (T) and frontal (U) views of a stage 14 embryo injected with *XBF-1*. Red staining represents co-injected *lacZ*. Black brackets indicate the size of the anterior expression domain in the uninjected embryo (P); white brackets indicate the size of the corresponding domain in the injected embryo (Q). Arrows in D,E,F,S,V indicate the anterior boundaries of *X-ngnr-1* expression; the arrow in T indicates *X-ngnr-1* ectopic expression. Black arrowheads in I,J indicate the continuous anterior extension of *X-ngnr-1* expression. White arrowheads in U and R indicate the repressed anterior expression domain of *X-ngnr-1*.

occupied by *Xrx1* (data not shown). Thus, the early *Xrx1* expression appears to be restricted, spatially and temporally, to the proliferative area of the anterior neural plate more specifically than that of other transcription factors. How is *Xrx1* expression localized to this area and how are its expression boundaries defined? We found that both positive and negative signals contribute to this positioning, as *Xrx1* is activated by *chordin* and Hedgehog and is repressed by retinoic acid and *X-ngnr-1*. As BMP antagonists are known to act as general forebrain inducers (Sasai and De Robertis, 1997), *chordin* may be responsible for the initial activation of *Xrx1* in the anterior neuroectoderm. Hedgehog signaling has been shown to delay differentiation and promote proliferation at early neurula stage (Franco et al., 1999); thus, the observation that Hedgehog signaling can activate *Xrx1* correlates with the expression of *Xrx1* in anterior proliferating cells. Different Hedgehog genes share the same activity, with regional specificity being achieved by differential expression of the family members. Thus, it is expected that *X-shh* and *X-chh* were equally effective in *Xrx1* activation, although it is likely that *X-chh*, which displays anterior expression (Ekker et al., 1995), assumes this function in normal development. At later stages, when the proximodistal axis of the diencephalon/eye-tract develops, the response of *Xrx1* to the Hedgehog gradient may become more complex. In fact, *Xrx1* is expressed in the retina and retinal pigmented epithelium, where the level of Hedgehog is low, is absent in the optic stalk, and is expressed again in the diencephalon floor, a region exposed to the highest level of Hedgehog signal. *Xrx1* expression in the anterior neural plate is also controlled by negative regulators such as RA and *X-ngnr-1*, which appear to prevent *Xrx1* expansion to surrounding regions. This is reflected in the spatial relationship between *Xrx1* on one side, and *XRALDH2*, a RA-producing enzyme (Chen et al., 2001), and *X-ngnr-1* on the other. *Xrx1* is not simply regulated by factors controlling neuronal differentiation, but plays an active role in this process. In fact, *Xrx1* is able to repress neuronal differentiation markers and to counteract the effects of *X-ngnr-1* and RA on neurogenesis. Both in the case of neurogenin and RA, *Xrx1* action appears to be twofold. *Xrx1* acts upstream by repressing *X-ngnr-1* and *XRALDH2* expression, and downstream or in parallel, by blocking the differentiation promoting activity of these factors. Although at early neurula stage, *XRALDH2* and *Xrx1* expression domains are adjacent but do not overlap, at later stages *XRALDH2* expression extends into the retina (Chen et al., 2001). This indicates that although at later stages RA is required for retina formation, for the initial specification of the eye field it is important to keep RA in the anterior neural plate at a very low level. The RA degrading activity of Cyp26 provides protection from RA coming from the posterior neural plate (Hollemann et al., 1998). However, the lack of expression of Cyp26 in the most

rostral region of the embryo leaves this area exposed to RA generated by the anterior domain of *XRALDH2*. *Xrx1*, the expression pattern of which is complementary to that of Cyp26 (data not shown), may be one of the elements that counteracts the RA signal in the anterior neural plate.

Besides counteracting neuronal differentiation, *Xrx1* promotes proliferation in the anterior neural plate. In fact, BrdU incorporation in gain- and loss-of-function experiments provides a direct evidence that *Xrx1* is both necessary and sufficient to regulate proliferation in the anterior neural plate. In particular, loss of *Xrx1* activity reduces anterior neural plate proliferation to levels similar to those observed in the posterior neural plate, indicating that *Xrx1* is one of the main factors responsible, directly or indirectly, for the increased proliferation of the anterior neural plate. So far, among the Rx genes that promote an enlargement of the retina, a proliferation inducing activity has been suggested for medaka *Rx3* (Loosli et al., 2001) but not for zebrafish *rx1* and *rx2* (Chuang and Raymond, 2001). Although species-specific differences may exist, the orthology relationship between vertebrate Rx genes has not yet been completely clarified.

Anterior-specific activities of *Xrx1*

We previously noticed that the phenotypic effects of *Xrx1* overexpression are restricted to the eye-anterior brain region, despite of the wider distribution of the injected RNA (Andreazzoli et al., 1999). In the present work, we find that *Xrx1* is able to induce proliferation and repress neuronal differentiation in an anterior-specific manner. As Xrx1 is a transcription factor, it presumably acts by regulating the expression of target genes. Interestingly, previous experiments have shown that *Xrx1* microinjection activates ectopic expression of *XBF-1* in the lateroanterior border of the neural plate (Andreazzoli et al., 1999) (Fig. 2G). As the effects of *Xrx1* on neurogenesis are also observed in regions where *XBF-1* cannot be activated by *Xrx1*, and the *Xrx1* expression domain is larger than that of *XBF-1*, additional factors are likely to be regulated by *Xrx1* in the anterior neural plate. Although *Xrx1* appears to be a transcriptional activator (Andreazzoli et al., 1999; Chuang and Raymond, 2001), its overexpression repressed *X-ngnr-1*, *X-Delta-1*, *N-tubulin*, *XRALDH2* and *p27Xic1*. Consistently, *Xrx1* activates *Zic2* and *Xhairy2*, two transcriptional repressors involved in delaying neuronal differentiation. As these two genes have an anterior expression domain that partially overlaps with that of *Xrx1*, they may mediate the repressive effects of *Xrx1* in this system. Interestingly, mouse *Rx1* can activate *Hes1*, a *hairy* homologue, during retinogenesis (Furukawa et al., 2000), indicating that genes of the Hairy family might be evolutionary conserved *Rx1* targets. Moreover, mutations in human *ZIC2* induce holoprosencephaly, and the mouse knockout of *Hes1* affects eye morphogenesis, phenotypes that are similar to those produced by the loss of function of the *Rx1* gene (Tomita et al., 1996; Mathers et al., 1997; Brown et al., 1998; Andreazzoli et al., 1999). However, as *Zic2* is not induced by *Xrx1* in animal caps, additional factors are likely required for *Zic2* activation. Both the ectopic activation of *Zic2* and *Xhairy2*, and the repression of *X-ngnr-1*, *X-Delta-1*, *N-tubulin*, *XRALDH2* and *p27Xic1* by *Xrx1* overexpression are restricted to the anterior neural plate, suggesting that only this region is competent to respond to *Xrx1*. *Xrx1* loss-of-function experiments resulted in reduction, but not abolishment, of *Xhairy2* and *Zic2* expression (Fig. 8Q; data not shown), indicating that *Xrx1* is not the only factor responsible for their anterior activation. Conversely, *X-ngnr-1* anterior expression was expanded medially, probably as a consequence of the reduction of the eye field. This phenotype, which is essentially reproduced in HUA-treated embryos, is consistent with a severely reduced anterior proliferation. Accordingly, the functional inactivation of *Xrx1* does not appear to be sufficient to induce widespread ectopic *X-ngnr-1* across the anterior neural plate, presumably because of the persistence of *Zic2* and *Xhairy2* expression. Co-injection experiments revealed that *XBF-1*, but not *Xhairy2*, is able to rescue the anterior expansion of *X-ngnr-1* observed in *MoXrx1*-injected embryos. These data indicate that *Xhairy2* cannot maintain a normal level of proliferation in the anterior neural plate in the absence of *Xrx1* function. The ability of *XBF-1* to re-establish a *X-ngnr-1*-free region suggests that *Xrx1* might work in part through *XBF-1* and/or that both genes control anterior neural plate proliferation acting on common regulators, as is the case for *p27Xic1*.

Lateral inhibition is not involved in *Xrx1* activities

An important mechanism used during development to prevent neuronal differentiation is lateral inhibition, a process mediated by transduction of the Notch signal. We considered the possibility that *Xrx1* might work by increasing lateral inhibition. This hypothesis was supported by the co-expression of *Xrx1* and *X-Notch-1* at early neurula and by data indicating that mouse *Rx1* activates *Notch* transcription during retinogenesis (Furukawa et al., 2000). By contrast, we did not find activation of *X-Notch-1* in *Xrx1*-injected embryos during early-mid neurulation (stages 13-18). Furthermore, *Xrx1* expression could not be stimulated by expression of a constitutively active form of *Notch* at early neurula stage. Another way in which lateral inhibition could be triggered is by overexpression of *Delta*, but this possibility could also be ruled out as *Xrx1* represses *X-Delta-1* expression, probably as a consequence of *X-ngnr-1* inhibition. Finally, we checked if *Xrx1* repression of neuronal differentiation could be prevented by blocking lateral inhibition. We observed that co-injection of *Xrx1* and an antimorphic form of Delta, known to block lateral inhibition, does not affect the ability of *Xrx1* of repressing neuronal differentiation in the anterior regions of the embryo.

These data suggest that *Xrx1* does not work through lateral inhibition involving Delta and Notch, but may bypass this system through the activation of *Xhairy2*, a target gene of *Notch* (Davis et al., 2001). In general, lateral inhibition is probably not responsible for preventing precocious neuronal differentiation in the anterior neural plate. In fact, the inability of *noggin*-injected animal caps, which display an anterior neuroectodermal character, to undergo neuronal differentiation at early neurula stage is not mediated by lateral inhibition (Papalopulu and Kintner, 1996). Similarly, the inhibition of neuronal differentiation after injection of high doses of *XBF-1* is not due to increased lateral inhibition (Bourguignon et al., 1998).

Distinct anterior and posterior gene systems control neuronal differentiation

In *Drosophila*, prepattern genes that are expressed before the

onset of neurogenesis control the region-specific activation of proneural genes. Prepattern genes include *hairy* and the Iroquois family homeobox genes (Gomez-Skarmeta et al., 1996; Fisher and Caudy, 1998). In vertebrates, homologues of the Iroquois genes play a similar role, functioning during early neurulation in the specification of neural precursors in the posterior neural plate (Bellefroid et al., 1998; Gomez-Skarmeta et al., 1998; de la Calle-Mustienes et al., 2002; Itoh et al., 2002). We notice several similarities between the activities of *Xenopus* Iroquois (*Xiro*) genes and *Xrx1*, as these genes: (1) repress neuronal differentiation at early neurula; (2) do not work through lateral inhibition; (3) are repressed by *X-ngnr-1* and activated by hedgehog signaling; (4) upregulate *Xhairy2* and *Zic2*; and (5) act after neural induction and before the selection of neuronal precursor cells.

Moreover, the loss of function of Rx genes in vertebrates as well as of the Iroquois complex in *Drosophila*, results in the absence of the structures where these genes are normally expressed (Cavodeassi et al., 2001; Mathers et al., 1997; Andreazzoli et al., 1999; Loosli et al., 2001). Beside these similarities, it is worth noting that while the Iroqouis genes play a role in positioning domains of proneural gene expression, this function has not been demonstrated for the Rx genes. However, the complementary expression of *Xrx1* and *Xiro* genes together with their similar activities suggest the existence of two gene systems, one acting in the anterior and the other in the posterior neural plate, the function of which is to control the timing and delimit the location of neuronal differentiation.

In conclusion, *Xrx1*, by counteracting differentiating signals and promoting proliferation in a region-specific manner, plays a crucial role in executing a program that, after neural induction, leads to the correct differentiation and patterning of the anterior neural plate.

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