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Functional association of retinoic acid and *hedgehog* signaling in *Xenopus* primary neurogenesis

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SUMMARY

Previous work has shown that the posteriorising agent retinoic acid can accelerate anterior neuronal differentiation in *Xenopus laevis* embryos (Papalopulu, N. and Kintner, C. (1996) *Development* 122, 3409-3418). To elucidate the role of retinoic acid in the primary neurogenesis cascade, we investigated whether retinoic acid treatment of whole embryos could change the spatial expression of a set of genes known to be involved in neurogenesis. We show that retinoic acid expands the *N-tubulin*, *X-ngnr-1*, *X-MyT1*, *X-Delta-1* and *Gli3* domains and inhibits the expression of *Zic2* and *sonic hedgehog* in the neural ectoderm, whereas a retinoid antagonist produces opposite changes. In contrast, *sonic* and *banded hedgehog* overexpression reduced the *N-tubulin* stripes, enlarged the neural plate at the expense of the neural crest, downregulated *Gli3* and upregulated *Zic2*. Thus, retinoic acid and *hedgehog* signaling have opposite effects on the prepattern genes *Gli3* and *Zic2* and on other genes acting downstream in the neurogenesis cascade. In addition, retinoic acid cannot rescue the inhibitory effect of *Notch^{ICD}*, *Zic2* or *sonic hedgehog* on primary neurogenesis. Our results suggest that retinoic acid acts very early, upstream of *sonic hedgehog*, and we propose a model for regulation of differentiation and proliferation in the neural plate, showing that retinoic acid might be activating primary neurogenesis by repressing *sonic hedgehog* expression.

Key words: Retinoic acid, *sonic hedgehog*, *banded hedgehog*, Primary neurogenesis, Neural patterning, *Xenopus laevis*

INTRODUCTION

In *Xenopus*, an early wave of neurogenesis along the posterior neural plate gives rise to *N-tubulin*-positive terminally differentiated primary neurons (medial, intermediate and lateral), which populate three longitudinal domains on each side of the dorsal midline (Chitnis et al., 1995). This process, known as primary neurogenesis, is under the control of proneural and neurogenic genes. In vertebrates, several genes producing transcription factors of the bHLH family, such as *XASH-3* and *X-ngnr-1* (Zimmerman et al., 1993; Ma et al., 1996), appear to be homologues of the *Drosophila* proneural genes and are thought to confer neuronal potential within each longitudinal domain. Neurogenic genes, such as the membrane-bound ligand *X-Delta-1* and its membrane-bound receptor *X-Notch-1*, limit the number of neuronal precursors by a process called lateral inhibition that controls the density of primary neuron formation within each proneural domain (Chitnis et al., 1995). In contrast, the zinc finger protein X-MyT1 allows cells to escape lateral inhibition, so they enter the pathway that leads to terminal neuronal differentiation (Bellefroid et al., 1996).

In *Drosophila*, the prepattern genes are distributed in domains larger than the proneural clusters and control the site-specific activation of the proneural genes (Gómez-Skarmeta et al., 1996). Molecules behaving as prepattern genes in vertebrates are *Zic2*, *Gli* genes (Brewster et al., 1998) and *Xiro* genes (Gómez-Skarmeta et al., 1998; Bellefroid et al., 1998). *Gli* genes are the vertebrate counterparts of *Drosophila cubitus interruptus (Ci)*, a zinc finger transcription factor gene that mediates the *hedgehog* signal (Alexandre et al., 1996; Domínguez et al., 1996). In *Xenopus*, as in other vertebrates, homologues of *Drosophila hedgehog* were isolated. In particular, *sonic hedgehog* (*X-shh*) is expressed by the notochord and the floor plate, and *banded hedgehog* (*X-bhh*) is expressed in the peripheral region of the neural plate and, later, at tadpole stages, in the roof plate and in the dermatome of the somites (Ekker et al., 1995). *Shh* induces floor plate cells and ventral motor neurons (Roelink et al., 1994, 1995; Martí et al., 1995; Tanabe et al., 1995; Hynes et al., 1995; Ericson et al., 1996), but the role of *Shh* or *Bhh* on primary neurogenesis has not been explored.

Endogenous retinoids are present in a posterior-to-anterior gradient in the early *Xenopus* embryo (Chen et al., 1994). Treatment with retinoic acid (RA) produces posteriorisations manifested as a concentration-dependent truncation of anterior structures and enlargement of posterior ones, anterior expansions of *Hox* genes domains and suppression of anterior neural markers (Durston et al., 1989; Sive et al., 1990; López

and Carrasco, 1992; Ang et al., 1994). In opposition, treatments with Ro 41-5253 (Ro), a selective and high-affinity antagonist of RARα and low-affinity antagonist of RARβ (Apfel et al., 1992), lead to a progressive reduction of the *Hoxb-7* and *Hoxc-6* domains, and to caudal expansions of *Krox-20* domains indicating anteriorisations (López et al., 1995 and unpublished results).

In *Xenopus*, *N-tubulin* expression anterior to the midbrain-hindbrain boundary is delayed until tailbud stages but RA induces premature anterior neuronal differentiation at the neurula stage (Papalopulu and Kintner, 1996). Hence, we wanted to determine whether RA can regulate primary neurogenesis in the posterior neural plate, where endogenous retinoid activity is maximum. We found that RA expands and Ro reduces the *N-tubulin* stripes of primary neurons. Therefore, we investigated whether RA and Ro treatments of whole embryos could alter the spatial expression of genes previously known to participate in the primary neurogenesis cascade. Our results show that RA expands the expression domains of positive regulators of neurogenesis (*X-ngnr-1*, *Gli3* and *X-MyT1*) and reduces the expression domain of *Zic2*, a negative regulator of primary neurogenesis (Brewster et al., 1998), whereas Ro produces opposite changes. Surprisingly, RA also inhibited *X-shh* expression in the dorsal midline. Therefore, we wanted to explore the effects of *X-shh* and *X-bhh* on primary neurogenesis. Overexpression of both *hedgehogs* produces a dramatic reduction of *N-tubulin* and expansion of *Zic2* together with an increase of cell number in the injected side. Finally, we show that the inhibition on *N-tubulin* expression produced by $Notch^{ICD}$, *Zic2* or *X-shh* injection could not be reverted by RA treatment, demonstrating that RA acts upstream of *X-shh* in the primary neurogenesis cascade. We propose a model where RA might be activating primary neurogenesis by the negative control of *X-shh* and suggest an important role of proliferation during patterning of the neural plate.

MATERIALS AND METHODS

Embryo culture, RNA injections and treatments

Albino *Xenopus laevis* embryos were obtained by in vitro fertilization using standard methods (Stern and Holland, 1992) and staged according to Nieuwkoop and Faber (1994).

Synthetic capped RNAs for microinjection were obtained by in vitro transcription using Megascript kit (Ambion) following the manufacturer instructions and were purified by Qiagen RNeasy mini kit. RNAs encoding *Zic2*, $Notch^{ICD}$, *X-shh* or *X-bhh* were coinjected with *nuc-βgal* RNA (100 pg) as tracer into one blastomere of 2-cell-stage embryos in 6% Ficoll, 1× MBS (Sive et al., 1996). The uninjected side was used as control. After 1 hour, embryos were cultured in 3% Ficoll, 0.1× MBS until sibling controls reached the desired stage. Early gastrulae (stage 9-10) were treated with all-*trans* RA (Sigma) or Ro 41-5253 (Roche) in 0.1× MBS until stage 15. Embryos were fixed with MEMFA (Harland, 1991).

For X-gal staining, fixed embryos were rinsed several times in PBS containing 0.1% Tween 20, washed 5 minutes in developing solution (7.2 mM Na_2HPO_4, 2.8 mM NaH_2PO_4, 150 mM NaCl, 0.1% Tween 20, 1 mM $MgCl_2$, 3 mM $K_3Fe(CN)_6$, 3 mM $K_4Fe(CN)_6$, pH 7.2) and transferred to fresh developing solution containing 0.027% X-gal for approximately 20 minutes at 37°C until adequate blue staining was achieved, then washed in PBS and stored in 100% ethanol at −20°C.

Whole-mount in situ hybridization, histological sections and Hoechst labeling

Templates for making antisense probes for in situ hybridization were linearised as follows: *N-tubulin* cDNA clone was digested with *Bam*HI and transcribed with T3; *X-MyT1* digested with *Cla*I, transcribed with T7; *X-ngnr-1* digested with *Bam*HI, transcribed with T3; *X-Delta-1* digested with *Xho*I, transcribed with T7; *Gli3* digested with *Bam*HI, transcribed with T7; *Zic2* digested with *Bam*HI, transcribed with SP6; *nrp1* digested with *Bam*HI, transcribed with T3; *Krox-20* digested with *Eco*RI, transcribed with T7; *Slug* digested with *Cla*I, transcribed with SP6,; *Xsal-1* digested with *Hin*dIII, transcribed with T7. The *X-shh* cDNA for in situ probes was independently isolated by Alejandra Paganelli from a *X. laevis* neurula cDNA library in λ-zap II and was digested with *Kpn*I transcribed with T3. Antisense RNA probes were prepared by in vitro transcription of the linearised DNA templates in the presence of digoxigenin-11-UTP (Boehringer-Mannheim).

To remove the unincorporated nucleotides, probes were purified using a Sephadex G25 coarse (Pharmacia) spin column prepared in TE pH 8.0, 0.1% SDS, and the eluate was ethanol precipitated. Whole-mount in situ hybridization was performed as previously described (Harland, 1991; Haramis and Carrasco, 1996), with some modifications. Once hydrated, embryos were treated with 2.5 μg/ml proteinase K (Merck) for approximately 10 minutes at room temperature, without manual removal of the vitelline membrane. The chromogenic reaction was developed with NBT and BCIP.

Histological 20 μm sections of Paraplast-embedded embryos were cut in a microtome. Nuclear labeling was performed in hydrated sections with 0.005% Hoechst 33258 (Polysciences) in 0.1× PBS for 10 minutes at room temperature.

RESULTS

RA increases and Ro decreases *N-tubulin* expression where primary neurogenesis takes place

In order to examine whether RA can regulate primary neurogenesis in the posterior neural plate, where endogenous retinoids display the highest activity, late blastulae were treated with RA and assayed for the expression of *N-tubulin* at neural plate stage by whole-mount in situ hybridization. At this time, *N-tubulin* is normally expressed in primary neurons organized in three longitudinal domains in the posterior neural plate: medial, intermediate and lateral, which correspond to motorneurons, interneurons and sensory neurons, respectively. A second site of expression is also detected in the trigeminal ganglia, near the midbrain-hindbrain boundary. Anterior *N-tubulin* expression does not begin before neural tube closure at tailbud stage (Papalopulu and Kintner, 1996; Fig. 1A).

RA strongly enhances the amount of *N-tubulin*-positive cells and signal intensity (Fig. 1B). Sensory neuron and interneuron stripes are so expanded that they collide. *N-tubulin* expression in the trigeminal placodes was lost, probably due to the posteriorising activity of RA.

To corroborate whether endogenous retinoids play a role in primary neurogenesis, we used the RARα and RARβ receptor antagonist Ro 41-5253 (Ro) as a tool to block retinoid mediated signaling. As expected, Ro produced a visible decline of *N-tubulin* expression in all three stripes (Fig. 1C).

These results indicate that endogenous retinoids can upregulate *N-tubulin* in vivo during primary neurogenesis. Since *N-tubulin* is a terminal neuronal differentiation marker

and one of the latest components of the neurogenesis cascade, we hypothesized that RA could be activating *N-tubulin* directly, or indirectly through other genes upstream of the cascade. To answer this question, we first explored which other molecules known to take part in the differentiation pathway upstream of *N-tubulin* can change their expression pattern after treatment with RA and Ro.

RA and Ro treatments alter the expression of genes upstream of *N-tubulin*

Embryos treated with RA or Ro were analyzed by in situ hybridization at neural plate stage with probes of different genes involved in the primary neurogenesis cascade.

X-MyT1, which is required for neuronal precursors to escape lateral inhibition, is normally expressed in the three longitudinal stripes where neurons will differentiate, in the trigeminal placodes and in a central anterior stripe (Bellefroid et al., 1996; Fig.1D). RA treatment enhanced the density of *X-MyT1*-expressing cells and merged the expanded interneuron and sensory neuron stripes, while shifting anteriorly the entire domain and suppressing the expression in the trigeminal placodes because of posteriorisation (Fig. 1E). Conversely, Ro reduced the longitudinal stripes while the expression in the most anterior domain was increased. Thus, retinoid regulation on *X-MyT1* expression is similar to that found with *N-tubulin*, but from these results we cannot discern whether this effect is direct or indirect.

Since *X-ngnr-1* is believed to operate as a proneural gene and its overexpression promotes widespread *X-MyT1* and *N-tubulin* activation (Bellefroid et al., 1996; Ma et al., 1996), we decided to assay the effect of RA and Ro on *X-ngnr-1* at neurula stage. *X-ngnr-1* is normally expressed in the trigeminal placodes and in the three longitudinal domains of primary neurogenesis but in broader stripes than genes downstream in the cascade (Ma et al., 1996; Fig. 1G). *X-ngnr-1* expression was increased by RA and, similarly to *N-tubulin*, stripes were merged and shifted anteriorly as previously shown for *N-tubulin* and *X-MyT1* (Fig. 1H). Conversely, Ro reduced *X-ngnr-1* expression in the longitudinal proneural domains and enlarged the trigeminal placode expression according to the anteriorising effect of Ro (Fig. 1I). These results suggest that *X-ngnr-1* could be one mediator of the RA-induced activation of *N-tubulin* and *X-MyT1*.

In conclusion, RA not only increases the density of neuronal precursors within each stripe of primary neurogenesis, suggesting an impairment of lateral inhibition, but also abolishes the spacing between stripes, which could reflect changes in the activity of prepattern genes thus directing the neural plate towards a uniform proneural territory. To further investigate this hypothesis, we analyzed the expression of the neurogenic gene *X-Delta-1* and the prepattern genes *Gli3* and *Zic2* after RA and Ro treatments.

The neurogenic gene *X-Delta-1* encodes a lateral inhibitory ligand that prevents neighboring cells from undertaking the neuronal fate. At neurula stage, *X-Delta-1* transcripts are normally found in the longitudinal domains where primary neuronal precursors arise, in the trigeminal placodes and in an anterior domain (Chitnis et al., 1995; Fig. 1J). While the expression in the trigeminal placodes and the most anterior domains were abolished by RA treatment due to posteriorisation, *X-Delta-1* expression in the posterior domains was raised, merged and shifted anteriorly as other markers described above (Fig. 1K). Ro changed the expression pattern of *X-Delta-1*, resolving the longitudinal stripes into a bilateral one towards the midline, while the anterior domains converged (Fig. 1L). Since *X-Delta-1* appears to be expressed in the future primary neurons themselves, we presume that the expansion of *X-Delta-1* domains upon RA treatment indicates that more cells are committed to neuronal differentiation.

Gli3 is expressed in the neural plate in a graded fashion, with highest levels in lateral regions and absent from the floor plate (Marine et al., 1997; Lee et al., 1997; Fig. 1M). *Gli3* has been shown to induce primary neurogenesis and to inhibit neural crest differentiation (Brewster et al., 1998). RA treatment notably increased *Gli3* expression in the posterior neural plate, now appearing uniform over the mediolateral axis, but remained absent from the floor plate, and the anterior domain was notoriously reduced (Fig. 1N). In contrast, Ro treatment reduced *Gli3* expression in the posterior neural plate but the anterior domain seemed unaffected (Fig. 1O). These results suggest that *Gli3* is activated by endogenous retinoids in the posterior neural plate and that *Gli3* could be one mediator of RA-enhanced neurogenesis.

Previous studies have shown that *Zic2* is expressed in stripes that alternate with those in which primary neurons differentiate and overlaps the domains of floor plate and neural crest progenitors (Brewster et al., 1998; Nakata et al., 1998); its overexpression forbids neuronal differentiation (Brewster et al., 1998). After RA treatments, *Zic2* expression shows a strikingly dose-dependent abolishment (Fig. 1Q). Interestingly, in Ro-treated embryos, *Zic2* expression was no longer restricted to the normal alternating striped pattern but became dispersed over the mediolateral axis in the posterior neural plate (Fig. 1R). We presume that the dispersion of *Zic2* may account for the repression of neurogenesis by Ro. Therefore, our results imply that endogenous retinoids can promote primary neurogenesis through the repression of *Zic2*.

In conclusion, endogenous retinoids act very early in the primary neurogenesis cascade and can regulate the activity of prepattern genes, promoting the expression of positive regulators of neurogenesis like *Gli3* and disfavoring the expression of negative regulators like *Zic2* but, again, we do not know whether this action is direct or through an upstream regulator of these genes.

Endogenous retinoids downregulate *X-shh* expression

Different concentrations of Shh induce floor plate cells and ventral neurons in vitro (Roelink et al., 1995) and ectopic expression of *Shh* within the neural tube of *Xenopus* embryos induces floor plate cells (Roelink et al., 1994). In *Drosophila*, the *Gli* family member *Ci* mediates the *hedgehog* signal (Domínguez et al., 1996). While *Gli1*, *Gli2* and *Gli3* promote primary neuron formation (Brewster et al., 1998), injection of *Shh* plasmids in *Xenopus* embryos ectopically activates *Gli1* and *Gli2* mostly outside the neural ectoderm but represses *Gli3* transcription in the neural plate (Lee et al., 1997; Ruiz i Altaba, 1998), suggesting a link between vertebrate *hedgehogs*, *Gli* genes and primary neurogenesis. However, a role for the *hedgehog* family in primary neurogenesis had not been explored until now.

Since RA can induce ectopic *Shh* expression in the anterior

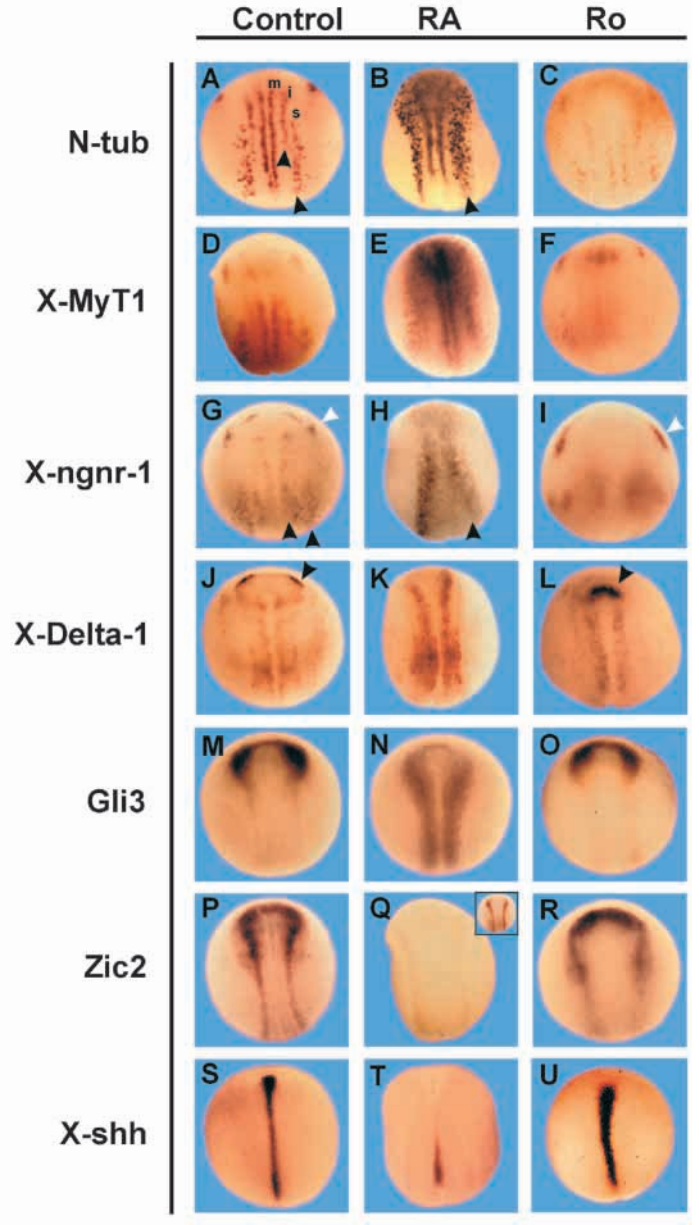

Fig. 1. RA exposure during gastrulation increased the expression of activators and repressed the expression of inhibitors of primary neurogenesis, while the retinoid antagonist Ro produced the opposite results. Embryos were untreated (Control column), treated with RA (RA column) or treated with Ro 41-5253 (Ro column) and the effect on the expression of different components of the primary neurogenesis cascade was evaluated by in situ hybridization at neurula stage. All panels are dorsal views (anterior up). (A-C) *N-tubulin* (N-tub) domains were strongly expanded by RA (B; 54%, *n*=24 for 1 μM RA; 100%, *n*=44 for 10 μM RA). The stripes of sensory neurons and interneurons were merged (compare arrowheads between A and B) and shifted anteriorly. Ro certainly reduced *N-tubulin* expression (C, 83%, *n*=12 for 1.5 μM Ro) and stripes appeared more distant than in control embryos. m, i and s, primary motor neurons, interneurons and sensory neurons, respectively. (D-F) *X-MyT1* domains were expanded and shifted anteriorly after RA treatment (E, 100%, *n*=8 for 1 μM RA; 100%, *n*=6 for 10 μM RA). Ro clearly reduced *X-MyT1* expression in the neural plate (F, 100%, *n*=8 for 1.5 μM Ro). (G-I) *X-ngnr-1* expression was increased, stripes were merged (compare black arrowheads in G and H) and shifted anteriorly, while trigeminal expression (white arrowhead in G) was lost in RA-treated embryos (H, 61%, *n*=33 for 1 μM RA; 72%, *n*=29 for 10 μM RA). Ro reduced *X-ngnr-1* expression in the neural plate but enlarged the trigeminal domain (white arrowhead; I, 21%, *n*=14 for 1.5 μM Ro; 20%, *n*=10 for 4 μM Ro). (J-L) *X-Delta-1* domains were increased, merged and shifted anteriorly in response to RA treatment, while the most-anterior domain (arrowhead in J) was lost probably due to posteriorization (K, 50%, *n*=16 for 10 μM RA, 38% *n*=16 for 1 μM RA). Ro changes the expression pattern of *X-Delta-1* resolving the longitudinal stripes into a bilateral one towards the dorsal midline, while the anterior domains (arrowhead) converge (L, 30%, *n*=10 for 1.5 μM Ro). (M-O) RA treatment resulted in a widespread expansion of *Gli3* over the mediolateral axis in the posterior neural plate, and the anterior domain was markedly reduced (N, 100%, *n*=10 for 1 μM RA; 100%, *n*=10 for 10 μM RA). Ro treatment only reduced the posterior expression (O, 67%, *n*=9 for 4 μM Ro; 63%, *n*=8 for 7.5 μM Ro). The low levels of Gli3 expression in the posterior domain in control embryos makes the comparison with Ro-treated embryos difficult. (P-R) RA produced a dose-dependent abolishment of *Zic2* expression (Q, complete lost, 100%, *n*=10 for 10 μM RA; posterior reduction and anterior lost, 100%, *n*=10 for 1 μM RA, see inset). Ro treatment dispersed *Zic2* expression over the mediolateral axis in the posterior neural plate (R, 30%, *n*=10 for 4 μM Ro; 40%, *n*=10 for 7.5 μM Ro). (S-U) *X-shh* expression was strongly reduced in the posterior level and was completely abolished in the anterior notochord and floor plate after RA treatment (T, 100%, *n*=23 for 10 μM RA). Ro-treated embryos showed a clear increase of *X-shh* along the dorsal midline (U).

margin of the chicken limb bud (Riddle et al., 1993) and our results suggest that endogenous retinoids promote primary neuron formation at a very early step in the cascade, we wanted to know whether endogenous retinoids could regulate *X-shh* expression at the time of primary neurogenesis. Therefore, we analysed the expression pattern of *X-shh* at neural plate stage after RA and Ro treatments.

In control embryos, *X-shh* transcripts are strongly expressed in the notochord and the floor plate at neurula stage (Fig. 1S). Surprisingly, RA completely abolished *X-shh* expression in the anterior half of the embryo and reduced the posterior expression (Fig. 1T). This observation was confirmed by transverse sections of these embryos (results not shown). Instead, Ro clearly increased *X-shh* expression along the dorsal midline (Fig. 1U). We conclude that endogenous retinoids downregulate *X-shh* when primary neurogenesis takes place.

Ectopic expression of *X-shh* and *X-bhh* suppresses primary neurons and enlarges the neural plate

Since RA restrained *X-shh* expression, we next questioned the role of *hedgehog* genes in primary neurogenesis. *X-shh* or *X-bhh* synthetic capped mRNAs were injected into one blastomere at the 2-cell stage and *N-tubulin* expression was revealed at stage 14-15. The injected side showed a downregulation in the three stripes of primary neurons and in the trigeminal ganglion (Fig. 2B,C). In some embryos, the sensory stripe was disorganized and displaced ventrally (Figs 2B,C, 3A). Therefore, we tested for a possible change in the size of the neural plate. A general neural marker, *nrp-1* (Knetch et al., 1995), showed an enlargement of the neural plate in the injected side (Fig. 2E,F). We conclude that both members of the *hedgehog* family can suppress primary neurogenesis but

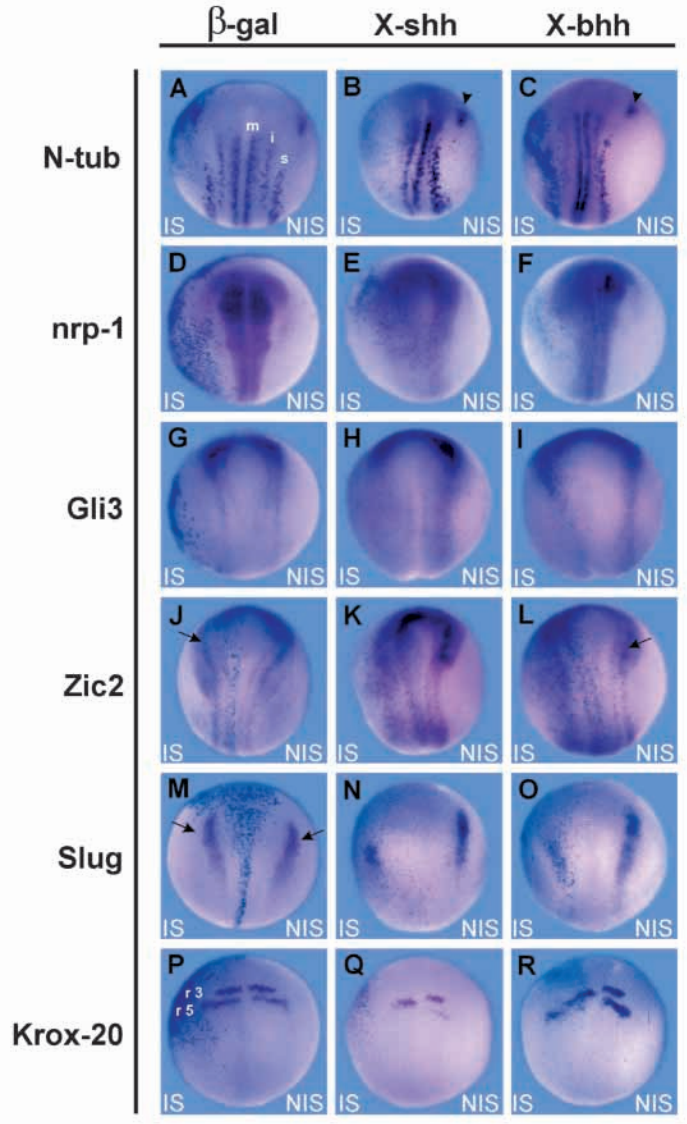

Fig. 2. *X-shh* and *X-bhh* overexpression increased the expression of inhibitors and repressed the expression of activators of primary neurogenesis, and reduced neural crest markers without impairing neural development. Embryos were unilaterally injected with 1 or 2 ng of *nuc-β galactosidase* mRNA as a negative control (*β-gal* column), full-length *X-shh* mRNA (*X-shh* column) or full-length *X-bhh* mRNA (*X-bhh* column) plus 100 pg of *nuc-βgal* mRNA as tracer. They were analyzed at neurula stage by whole-mount in situ hybridization with different neural markers. All are dorsal views (anterior up). The injected side is demarcated by the pale blue staining and is oriented to the left. IS, injected side. NIS, non-injected side. (A-C) Suppression of primary neuron formation as revealed by the differentiation marker *N-tubulin* (N-tub) in *X-shh*-injected embryos (B, 90%, *n*=21 for 2 ng; 79%, *n*=14 for 1 ng; 57%, *n*=21 for 0.125 ng) and *X-bhh*-injected embryos (C, 100%, *n*=24 for 2 ng; 93%, *n*=14 for 1 ng; 28%, *n*=22 for 0.125 ng). Notice the absence of *N-tubulin* expression from the trigeminal ganglion in the injected side. m, i and s, primary motor neurons, interneurons and sensory neurons, respectively; arrowhead, trigeminal ganglion. (D-F) Expansion of the neural plate as revealed by the general neural marker *nrp-1* in *X-shh*-injected embryos (E, 100%, *n*=10 for 2 ng) and *X-bhh*-injected embryos (F, 100%, *n*=10 for 2 ng). (G-I) Abolishment of *Gli3* expression in the posterior neural plate in *X-shh*-injected embryos (H, 67%, *n*=21 for 2 ng; 15%, *n*=13 for 1 ng) and *X-bhh*-injected embryos (I, 50%, *n*=26 for 2 ng). Notice that nuc-β-gal dark-blue staining may interfere with the appreciation of Gli3 decrease on the injected side. (J-L) Widespread expansion of *Zic2* domain in *X-shh*-injected embryos (K, 71%, *n*=34 for 2 ng; 44%, *n*=18 for 0.25 ng) and *X-bhh*-injected embryos (L, 80%, *n*=35 for 2 ng; 32%, *n*=63 for 0.25 ng). Notice the absence of *Zic2* expression from the medial cranial neural crest domain (arrow) in the injected side. (M-O) Reduction and ventral displacement of the *Slug* domain in *X-shh*-injected embryos (N, 82%, *n*=22 for 2 ng; 64%, *n*=11 for 1 ng; 35%, *n*=17 for 0.25 ng) and *X-bhh*-injected embryos (O, 50%, *n*=6 for 1 ng; 32%, *n*=25 for 0.25 ng). Arrows, neural crests. (P-R) Downregulation of *Krox-20* in r5 and caudal displacement of r3 domain in *X-shh*-injected embryos (Q, 60%, *n*=10 for 2 ng) and *X-bhh*-injected embryos (R, 60%, *n*=10 for 2 ng). r3, third rhombomere; r5, fifth rhombomere.

this in not due to an impairment of neural development. Moreover, they increase the size of the neural plate.

Zic2 is activated and *Gli3* is inhibited by *X-shh* and *X-bhh* overexpression

To understand whether prepattern genes mediate the suppression of primary neurogenesis by *hedgehog* signals, we examined the effects of *X-shh* and *X-bhh* overexpression on *Gli3* and *Zic2*. Both family members produced similar results. We observed a fade out of *Gli3* (Fig. 2H,I), as was previously shown for *Shh* overexpression (Ruiz i Altaba, 1998). The *Zic2* domain is certainly expanded over the injected side in the neural plate, i. e., in the posterior neural folds and the stripes of the posterior neural plate and in most of the anterior neural fold, including the lateral cranial neural crest (for a detailed description of neural crest development in *Xenopus laevis* embryos, see Sadaghiani and Thiébaud, 1987). However, the domain of the medial cranial neural crest no longer expresses *Zic2* (Fig. 2K,L), suggesting an inhibition of neural crest fate in a subset of cells in the anterior neural fold (see below).

Cross sections of injected embryos clearly reveal the unilateral upregulation of *Zic2*. Interestingly, in the neural ectoderm, *Zic2* transcripts were found in the nuclei of the injected side, indicating a very active transcription (Fig. 3C-E). A clear expansion of the neural ectoderm and the somitogenic and lateral mesoderm is also evident due to a strong increase in the amount of cells on the injected side (Fig. 3B,C).

In conclusion, *X-shh* and *X-bhh* overexpression may be suppressing primary neurogenesis by restraining the activity of prepattern genes that promote primary neuron formation like *Gli3*, and expanding the domains of inhibitors of this differentiation pathway like *Zic2*.

X-shh and *X-bhh* overexpression affects the cranial neural crest and rhombomeric patterning

Because overexpression of *X-shh* and *X-bhh* enlarged the neural plate, displaced ventrally the sensory stripe of primary neurons and reduced the trigeminal ganglion, we used *Slug* as a marker to see if they could be impairing neural crest development (Mayor et al., 1995; Fig. 2M). In the injected side, the expression of *Slug* was absent or reduced and displaced to more ventral positions (Fig. 2N,O), in agreement with the expansion of the neural plate. Coincidentally, in tadpoles, we observed a downregulation of *Xsal-1* (Hollemann et al., 1996)

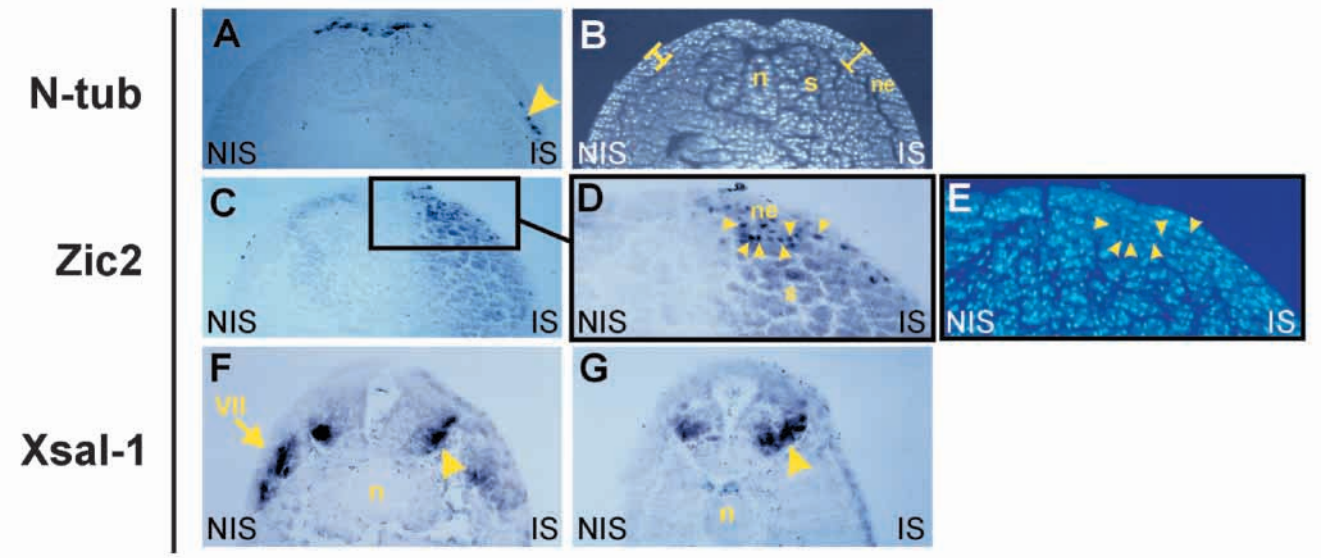

Fig. 3. Cross sections of *X-shh*- and *X-bhh*-injected embryos at neurula stage (A-E) and tadpole stages (F,G). Dorsal side is up. IS, injected side; NIS, non-injected side; n, notochord; ne, neural ectoderm; s, somites. (A) *N-tubulin* distribution shows the ventral displacement of the primary sensory neurons stripe (arrowhead) in the IS. (B) Hoechst nuclear labeling revealing the increased cell number in the IS including the neural ectoderm (compare bars). (C,D) *Zic2* expression. Note the expansion in the neural ectoderm and mesoderm in the IS in C. The inset shown at higher magnification in D shows the very active transcription of *Zic2* in nuclei (arrowheads) of the IS. (E) The same section as in D revealed for Hoescht staining, confirms the nuclear location (arrowheads) of *Zic2* transcripts. (F,G) *Xsal-1* expression is downregulated in the VIIth cranial ganglion (arrow in F) and reveals an expansion of ventral secondary neurons within the neural tube (arrowheads in F and G) in the IS.

in the ganglion of the VIIth cranial nerve, another neural crest derivative (Fig. 3F).

When embryos were probed with *Krox-20*, a marker for rhombomeres r3 and r5 and their corresponding migrating neural crest in the third visceral arch mesenchyme (Bradley et al., 1992; Fig. 2P), we observed a downregulation in r5 and a caudal displacement of r3 (Fig. 2Q,R), indicating that the overexpression of *X-shh* and *X-bhh* not only downregulates *Krox-20*, but also affects the rhombomeric patterning.

We conclude that *X-shh* and *X-bhh* overexpression expands the neural plate at the expense of neural crest development and also impairs anteroposterior patterning in the hindbrain.

X-shh and *X-bhh* regulate secondary neurogenesis

Several reports revealed that *Shh* is required for ventral neuron formation (Roelink et al., 1994, 1995; Tanabe et al., 1995; Hynes et al., 1995; Ericson et al., 1996; Chiang et al., 1996). As we have found that both *X-shh* and *X-bhh* can suppress primary neuron formation, we explored their effect on secondary neurogenesis. We examined the expression pattern of *Xsal-1* in *X-shh*- and *X-bhh*-injected tadpoles (stage 32). In the spinal cord of control embryos, *Xsal-1* is confined to motor neurons and interneurons, and also is expressed in the ganglion of the VIIth cranial nerve (Hollemann et al., 1996). In cross sections, the neural tube of injected embryos showed an expansion of the *Xsal-1* ventral domain (Fig. 3F,G) and an increase of cell number detected by nuclear Hoechst staining (data not shown). These results indicate that *X-shh* and *X-bhh* overexpression can promote secondary differentiation of ventral neurons. Outside the central nervous system, *Xsal-1* was downregulated in the ganglion of the VIIth cranial nerve (Fig. 3F), perhaps due to the inhibition of neural crest development.

RA treatments cannot rescue the inhibitory effect of *Notch^{ICD}*, *Zic2* and *X-shh* on primary neurogenesis

We have shown that RA promotes primary neuron differentiation by regulating several components of the cascade. However, from our previous results, we could not discern between a direct action all over the cascade or only on upstream genes. To understand at what steps RA is required, we explored whether RA treatment could overcome the effect of inhibitory molecules acting at different levels in the differentiation pathway. We injected embryos in one blastomere at the 2-cell stage with synthetic capped mRNAs encoding *Notch^{ICD}* (a constitutively active form of *X-Notch* that blocks *N-tubulin* expression by triggering lateral inhibition; Chitnis et al., 1995), *Zic2* or *X-shh*, treated them with RA, and analyzed the *N-tubulin* distribution at neurula stage.

Despite RA treatment, *N-tubulin* was clearly reduced or absent from the *Notch^{ICD}*-, *Zic2*- or *X-shh*-injected sides (Fig. 4). These results indicate that RA must be acting upstream of lateral inhibition, *Zic2* and *X-shh* in the neurogenesis cascade.

DISCUSSION

To understand the role of RA and members of the *hedgehog* family in primary neurogenesis, we analyzed their effects on the expression of different genes involved in the neurogenesis cascade in whole *Xenopus laevis* embryos.

Endogenous retinoids activate the expression of genes that promote and reduce the expression of genes that inhibit primary neurogenesis

Previous work demonstrated that RA treatment can accelerate neuronal differentiation in the anterior neural plate of whole

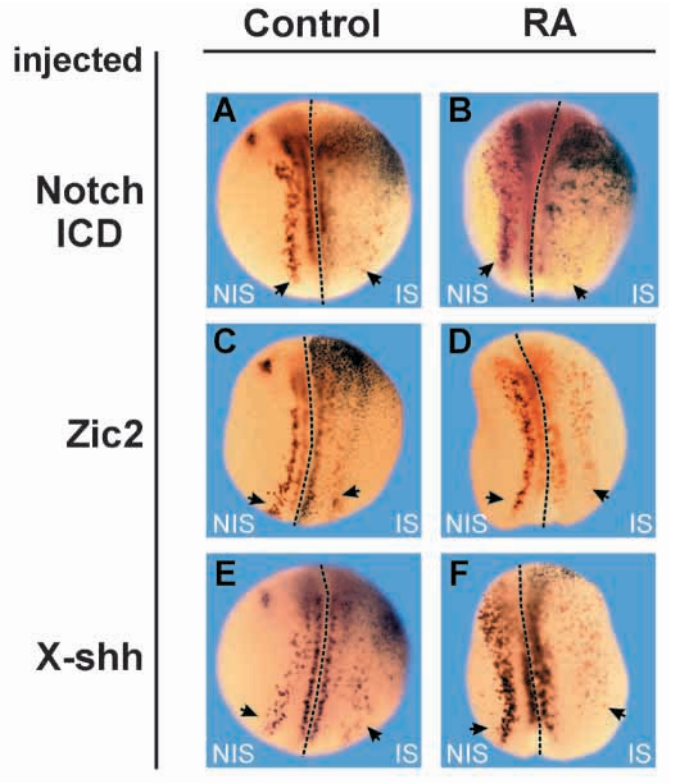

Fig. 4. RA acts upstream of lateral inhibition, *Zic2* and *X-shh* in the primary neurogenesis cascade. Embryos were coinjected unilaterally with 100 pg *nuc-β-gal* mRNA as tracer plus $Notch^{ICD}$, *Zic2* or *X-shh* mRNAs and left untreated (control column) or treated with RA during gastrulation (RA column). *N-tubulin* distribution was revealed by in situ hybridization at neurula stage. All are dorsal views (anterior up). Dotted line, dorsal midline; IS, injected side (blue staining); NIS, non-injected side. Sensory neuron stripes are marked with arrows. Percentages below indicate the reduction of *N-tubulin* expression in the IS. (A,B) RA treatment cannot overcome the inhibitory effect of $Notch^{ICD}$ on *N-tubulin* expression. $Notch^{ICD}$-injected embryos, untreated (A, 93%, *n*=11 for 1 ng $Notch^{ICD}$; 75%, *n*=14 for 0.5 ng $Notch^{ICD}$). $Notch^{ICD}$-injected embryos, treated with RA (B, 100%, *n*=10 for 1 ng $Notch^{ICD}$ + 10 μM RA; 70%, *n*=10 for 1 ng $Notch^{ICD}$ + 1 μM RA; 73%, *n*=11 for 0.5 ng $Notch^{ICD}$ + 10 μM RA; 100%, *n*=10 for 0.5 ng $Notch^{ICD}$ + 1 μM RA). (C,D) RA treatment cannot rescue the inhibitory effect of *Zic2* on *N-tubulin* expression. *Zic2*-injected embryos, untreated (C, 20%, *n*=22 for 2 ng *Zic2*; 15%, *n*=32 for 1 ng *Zic2*). *Zic2*-injected embryos, treated with RA (D, 22%, *n*=23 for 1 ng *Zic2* + 10 μM RA). (E,F) RA treatment cannot rescue the inhibitory effect of *X-shh* on *N-tubulin* expression. *X-shh*-injected embryos, untreated (E, 17%, *n*=6 for 0.25 ng *X-shh*). *X-shh*-injected embryos, treated with RA (F, 30%, *n*=10 for 0.25 ng *X-shh* + 10 μM RA). All RA-treated embryos showed the previously described enhancement of *N-tubulin* expression in the uninjected side.

embryos (Papalopulu and Kintner, 1996). Could RA also alter neuronal differentiation in the posterior neural plate where endogenous RA might mainly play its role and where primary neurogenesis occurs?

Here we showed that RA exposure during gastrulation greatly expanded the normal domains of *N-tubulin* expression at neural plate stage. In contrast, Ro treatments decreased *N-tubulin* expression, in agreement with the loss of primary neurons produced by the microinjection of dominant negative forms of retinoic acid receptors (Blumberg et al., 1997, Sharpe and Goldstone, 1997).

We also show that RA treatment increased the domains of genes previously shown to promote neuronal differentiation such as *X-ngnr-1*, *X-MyT1* and *Gli3*. The deletion of spacing between the stripes of *X-ngnr-1* and *X-MyT1* suggested that RA was changing the activity of prepattern genes, thus directing the neural plate towards a uniform proneural territory. Indeed, RA produced a widespread *Gli3* expansion in the posterior neural plate and a dramatic downregulation of *Zic2*, a gene proposed to inhibit neuronal differentiation. The involvement of endogenous retinoids in this regulatory hierarchy was confirmed by blocking RA signaling with Ro, which produced opposite changes in the expression patterns of these genes.

Because *X-Delta-1* appears to be expressed in the future primary neurons themselves, they should be the source of the inhibitory signal that activates *X-Notch-1* in the neighboring cells, thus preventing them from undergoing neuronal differentiation, inhibiting their own *X-Delta-1* expression and decreasing their ability to inhibit the original signaling cell. This would generate a feedback loop that reinforces contrasts between adjacent cells (Chitnis et al., 1995). Here we showed that RA treatment enhanced the density of *X-Delta-1*-positive cells and we presume that, in this way, impaired the contrasts between adjacent cells, allowing more precursors to become neurons. Since *X-ngnr-1* overexpression leads to *X-Delta-1* overproduction (Ma et al., 1996), RA could be activating *X-Delta-1* expression through *X-ngnr-1* induction.

We also presented evidences that endogenous retinoids downregulate the expression of genes that inhibit neurogenesis,

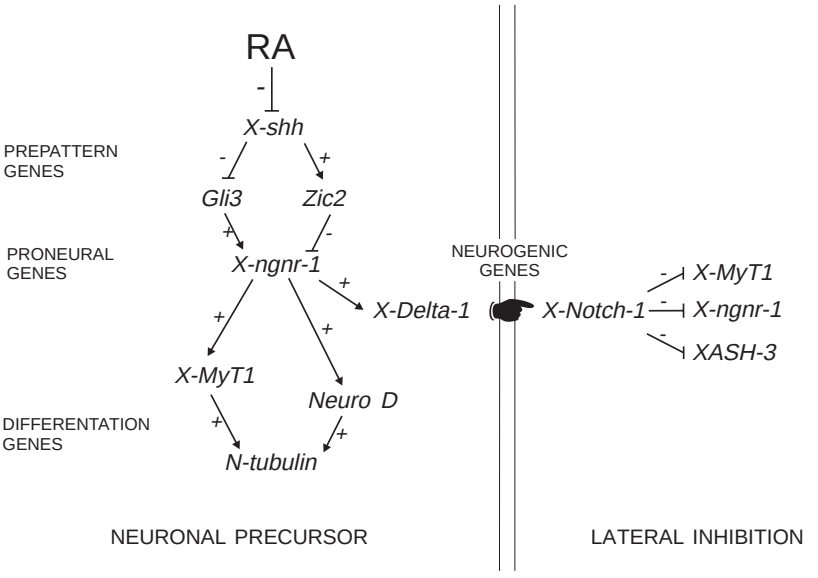

Fig. 5. Proposed model for the molecular interactions involving RA and *hedgehog* signaling leading to terminal primary neuronal differentiation.

like *Zic2* and *X-shh*. While RA treatment reduced their expression, after blocking RA signaling, *X-shh* expression was increased along the dorsal midline and *Zic2* expression became dispersed over the mediolateral axis of the neural plate, accounting for the inhibition of primary neurogenesis by Ro.

As previous work in chicken limb and zebrafish fin buds demonstrated an induction of *Shh* expression in response to RA (Helms et al., 1994; Chang et al., 1997; Niswander et al., 1994), we were surprised that *X-shh* expression was downregulated by RA at neurula stage both in the notochord and floor plate. These results agree with the very early transient downregulation observed in developing and regenerating axolotl limbs (Torok et al., 1999). Furthermore, the upstream region of zebrafish *shh* contains a retinoic acid responsive element (RARE), implying a direct regulation of the *shh* gene by RA (Chang et al., 1997). These reports and our results clearly add evidence for a link between *X-shh* and RA at the molecular level.

X-shh and *X-bhh* suppress primary neurogenesis, increase secondary neurogenesis and might promote proliferation

The suppression of primary neurogenesis produced by the overexpression of *X-shh* and *X-bhh* was not due to inhibition of neural development, because the neural plate was expanded on the injected side, as shown with the general neural marker *nrp-1*. When compared to RA treatments, *X-shh* and *X-bhh* overexpression produced opposite changes in the expression patterns of different members of the neurogenesis cascade that resembled Ro effects, suggesting that a counterbalance exists between retinoid and *hedgehog* signaling to restrict primary neurogenesis to the normal sites.

Precursors of the primary and secondary neurons arise from different layers of the neural plate. The superficial layer contains predominantly secondary precursors, whereas the deep layer contains both types of precursors at a similar density. (Hartenstein, 1989). Although we have not followed the fate of the cells inhibited to differentiate by *X-shh* and *X-bhh*, they probably participate in subsequent waves of neurogenesis, as suggested by the fact that both *hedgehog* members later expanded the number of cells expressing *Xsal-1*, a marker of ventral motor and intermediate neurons in the neural tube of tadpoles.

The evident expansion of the neural ectoderm and the paraxial mesoderm together with the increase in cell number are consistent with *X-shh* and *X-bhh* playing a proliferative role in both germ layers, but we cannot exclude an inhibition of cell death. Indeed, *Shh* promotes proliferation in the sclerotome (Johnson et al., 1994) and was recently reported to prevent differentiation and induce a proliferative response in cerebellar cells (Wechsler-Reya and Scott, 1999).

Therefore, we propose that both *hedgehog* members produce a differential effect on primary and secondary neuronal precursors, perhaps withdrawing cells from premature differentiation, holding their proliferative state and precluding them from subsequent waves of neuron formation.

RA acts upstream of *X-shh* in the neurogenesis cascade

We have shown that RA downregulated *X-shh* expression whereas Ro produced the opposite change. We propose that, in the normal embryo, *X-shh* expression in the dorsal midline should be controlled by positive and negative regulators. When negative regulation of *X-shh* is impaired by Ro, the equilibrium is displaced towards a gain-of-function of *shh* that correlates with decreased primary neuron differentiation.

Because RA treatments could not rescue the inhibitory effect of *X-shh* on neuronal differentiation, while *X-shh* overexpression produced a widespread expansion of *Zic2* and suppressed *Gli3*, we can suggest a cascade of interactions where endogenous retinoids act very upstream, promoting primary neurogenesis by inhibiting *X-shh* expression in the dorsal midline (Fig. 5). This in turn changes the balance of prepattern genes (activation of *Gli3* and reduction of *Zic2*), thus altering the expression of other intermediary genes, ultimately leading to *N-tubulin* activation.

Because in the normal embryo *X-shh* is expressed along the dorsal midline, it is evident that endogenous retinoids do not completely block *shh* signaling. This fact suggests that a precise balance between retinoid and *hedgehog* signaling must be established, resulting in the normal primary neurogenesis pattern. While endogenous retinoids constitute an early signal that promotes primary neuron formation by inclining the entire neural plate towards a uniform proneural territory, *shh* signaling is necessarily required at the same time and at an accurate level, limited at least by endogenous retinoids, to save a pool of neuronal precursors from premature differentiation by retinoid signaling, keeping them in a mitotic, undifferentiated state for subsequent waves of neurogenesis.

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