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A posteriorising factor, retinoic acid, reveals that anteroposterior patterning controls the timing of neuronal differentiation in *Xenopus* neuroectoderm

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SUMMARY

During early development of the *Xenopus* central nervous system (CNS), neuronal differentiation can be detected posteriorly at neural plate stages but is delayed anteriorly until after neural tube closure. A similar delay in neuronal differentiation also occurs in the anterior neural tissue that forms in vitro when isolated ectoderm is treated with the neural inducer *noggin*. Here we examine the factors that control the timing of neuronal differentiation both in embryos and in neural tissue induced by *noggin* (*noggin* caps). We show that the delay in neuronal differentiation that occurs in *noggin* caps cannot be overcome by inhibiting the activity of the neurogenic gene, *X-Delta-1*, which normally inhibits neuronal differentiation, suggesting that it represents a novel level of regulation. Conversely, we show that the timing of neuronal differentiation can be changed from late to early after treating *noggin* caps or embryos with retinoic acid (RA), a putative posteriorising agent. Concommittal with changes in the timing of neuronal differentiation, RA suppresses the expression of anterior neural genes and promotes the expression of posterior neural genes. The level of early neuronal differentiation induced by RA alone is greatly increased by the additional expression of the proneural gene, *XASH3*. These results indicate that early neuronal differentiation in neuralised ectoderm requires posteriorising signals, as well as signals that promote the activity of proneural genes such as *XASH3*. In addition, these result suggest that neuronal differentiation is controlled by anteroposterior (A-P) patterning, which exerts a temporal control on the onset of neuronal differentiation.

Key words: posteriorising factor, retinoic acid, anteroposterior patterning, neuronal differentiation, *Xenopus*, neuroectoderm

INTRODUCTION

The development of the vertebrate CNS begins when one portion of the embryo, the ectoderm, gives rise to the neural plate rather than differentiating into epidermis. These early events in CNS development require inductive interactions between the ectoderm and a region of the embryo called Spemann's organiser. Among the signals that appear to be generated by the organiser tissue are ones that neuralise the ectoderm while others appear to pattern the neuralised ectoderm along its A-P and dorsoventral (D-V) axes (reviewed in Doniach, 1993; Ruiz i Altaba, 1994).

In amphibian embryos, the signals underlying neural induction have been studied using animal cap assays in which ectoderm differentiates into epidermis but not neural tissue if isolated from blastula embryos and placed in culture. Isolated ectoderm can then be used to assay potential neural inducers by adding them to the culture media, or by expressing them in the ectoderm by injecting their RNAs at early cleavage stages. Using this assay, several molecules have been recently identified in *Xenopus* embryos that appear to act as bonafide neural inducers (reviewed in Harland, 1994). Noggin, a small secreted protein, can induce ectoderm to form neural tissue, either when expressed from an injected RNA or added exogenously as a purified protein to isolated ectoderm (Lamb et al., 1993; Knecht et al., 1995). Ectoderm will also form neural tissue when expressing follistatin, chordin, a truncated type II activin receptor, a truncated Bone Morphogenetic Protein (BMP) type I receptor, or a dominant negative BMP ligand (Hemmati-Brivanlou et al., 1994; Hemmati-Brivanlou and Melton, 1994; Hawley et al., 1995; Sasai et al., 1995; Xu et al., 1995). These agents inhibit signalling of TGF-β-like growth factors, most likely BMPs, which promote epidermal differentiation (Sasai et al., 1995; Wilson and Hemmati-Brivanlou, 1995; Hawley et al., 1995). Thus, one type of signal that is produced by the organiser is one that can neuralise ectoderm by blocking an epidermalising signal.

When ectoderm is neuralized with these various neural inducers, it forms neural tissue characterised as forebrain-like, while lacking most the features associated with other regions of the CNS (reviewed in Harland, 1994; see also Hawley et al., 1995; Sasai et al., 1995; Xu et al., 1995). Thus, it expresses anterior markers such as *Xotx2*, but fails to express significant levels of such midbrain markers as *En-2*, hindbrain markers such as *Krox-20* and spinal cord markers such as the *Hox* genes. In addition, some forebrain structures such as eyes are missing in this neural tissue. Finally, the same neural tissue shows a marked delay in neuronal differentiation, which can be followed by the expression of type II neuronal-specific

tubulin gene (hereafter referred to *N-tubulin*). Thus, while *N-tubulin* is expressed by primary neurons, which appear in discreet regions of the neural plate soon after gastrulation (Hartenstein, 1989; Chitnis et al., 1995), it is not detected in neural tissue induced in animal caps by *noggin* RNA until tadpole stages (Lamb et al., 1993). These observations then raise the question of what other signals in addition to *noggin* or similar inducers are required during neural induction to generate other regions along the vertebrate neural axis and to induce neuronal differentiation at early stages.

In this paper, we examine the factors that control neuronal differentiation along the A-P axis of the *Xenopus* embryo. We show that in embryos the timing of neuronal differentiation, as marked by *N-tubulin* expression, occurs posteriorly at the neural plate stage while anteriorly it is delayed until the tadpole stages. We then show that the delay in neuronal differentiation that also occurs in neural tissue induced in animal caps by *noggin* (*noggin* caps) cannot be overcome by expressing the proneural gene *XASH3*, which promotes neuronal differentiation in vitro and in vivo, or by inhibiting the activity of the neurogenic ligand, *X-Delta-1*, which normally inhibits neuronal differentiation (Ferreiro et al., 1994; Chitnis et al., 1995; Chitnis and Kintner, 1996). These findings suggested a novel level of regulation and led us to hypothesise that the timing of neurogenesis in *noggin* caps reflects the anterior character of *noggin*-induced neural tissue.

To test this hypothesis, we asked whether the timing of neuronal differentiation in noggin caps could be changed by treatment with RA. Previous studies have implicated RA in neural patterning, for instance by showing that RA treatment of whole embryos suppresses anterior and enhances posterior development (reviewed in Maden and Holder, 1992 and references within). We found that *noggin* caps treated with RA lose expression of anterior neural markers and gain expression of posterior neural markers, as expected for a posteriorising signal. At the same time, treatment of *noggin* caps with RA initiated early neuronal differentiation, and this effect was further stimulated by the additional expression of *XASH3*. In a similar manner, RA was also found to affect patterning and neurogenesis within the neural plate in vivo. Finally we show that the ability of RA to posteriorise and promote early neurogenesis in a *noggin* cap declines sharply by the end of gastrulation (stage 12.5), suggesting that the effects of RA on patterning and neuronal differentiation are closely coupled. From these results, we propose that neuronal differentiation in neuralized ectoderm requires posteriorising signals such as RA, as well as signals that promote the activity of proneural genes such as *XASH3*.

MATERIALS AND METHODS

Embryo culture, injections, dissections and treatment

Embryos were obtained from *Xenopus laevis* adult frogs by hormone-induced egg laying and in vitro fertilisation using standard methods. Embryos were staged according to (Nieuwkoop and Faber, 1967). Both blastomeres of 2-cell-stage embryos were injected with capped, synthetic RNAs encoding *noggin* (0.5 ng), *XASH3* (1.0 ng), *X-Delta-1*^{stu}, or a combination, as appropriate. RNA was prepared in vitro using SP6 RNA polymerase. *Noggin* mRNA (Lamb et al., 1993) was generated from a PCR clone containing the noggin coding sequence subcloned in pSP64T (N. Papalopulu, unpublished data). *XASH3* and *X-Delta-1*^{stu} RNA was transcribed from clones previously described (Ferreiro et al., 1994; Chitnis et al., 1995). Injected embryos were reared in 0.5× MMR (Kimelman and Kirschner, 1987) and animal caps were dissected at stage 9. Some animal caps and embryos were treated with 2×10^{-6} M RA, diluted in 0.5 or 0.1× MMR respectively, from a 10^{-2} stock of RA in DMSO, either shortly after dissection or at stage 12.5. Animal caps were cultured in 1% agarose dishes in 0.5× MMR containing penicillin/streptomycin (plus or minus RA) until sibling controls reached either neural plate (stage 16) or tadpole stage (stage 27), at which point they were either processed for RNAse protection analysis or fixed for in situ hybridisation. Other animal caps were dissected at stage 8, immediately treated with 200 pM recombinant activin in 0.5× MMR, 0.1% BSA and isolated for RNA analysis at stage 11.

In situ hybridisation

Embryos were fixed and processed as described in the in situ hybridisation protocol of Harland (1991). RNA probes were prepared by in vitro transcription of the linearised DNA templates in the presence of digoxigenin-11-UTP (Boehringer Mannheim). The probe for *Xotx2* is produced by linearising a 2.3 kb cDNA clone, pXOT30, with *Not*I

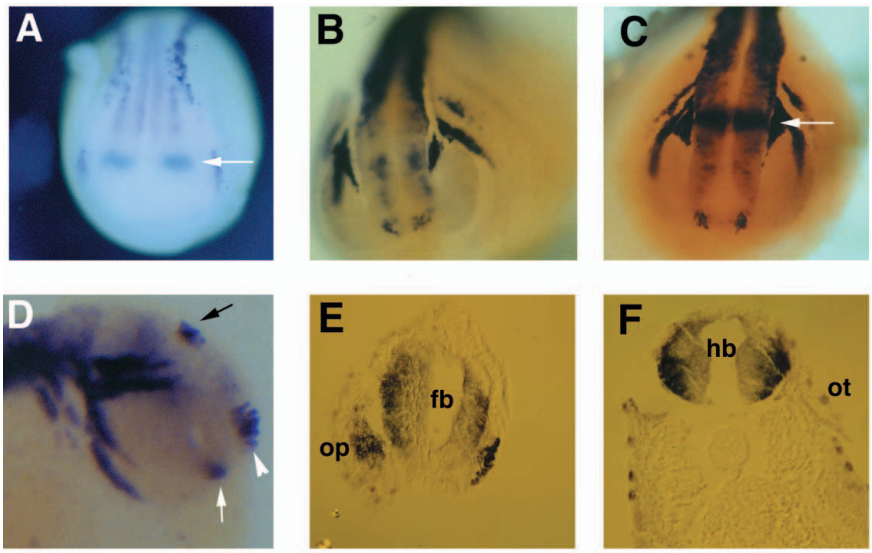

Fig. 1. Expression of the neuronal differentiation marker *N-tubulin*, in stage 16 neural plate (A), stage 27 (B,C,D) and stage 31 (E,F) tadpole *Xenopus* embryos. (A-D) Embryos analysed for *N-tubulin* expression shown in whole mount; (E,F) *N-tubulin* staining in sections. (A,C) Hybridised with *En-2*, which is a marker for the midbrain-hindbrain boundary and is shown with an arrow. (A-C) Frontal views; (D) side view of the head. Neuronal differentiation takes place at the neural plate stage and is confined to three stripes on either side of the dorsal midline. Note that the expression of *N-tubulin* is not detected anterior to *En-2* at the neural plate stage. In contrast, *N-tubulin* expression can be detected in the forebrain, starting at later tadpole stages (stage 27) when it is localised to the epiphysis (black arrow), ventral postoptic diencephalon (white arrow) and olfactory placodes (white arrowhead; see also Hartenstein, 1993). At stage 31, *N-tubulin* expression is abundant posteriorly (F) as well as anteriorly (E). (E) A section through the forebrain (fb), including olfactory placodes (op); (F) a section through the hindbrain (hb) at the level of the otocysts (ot).

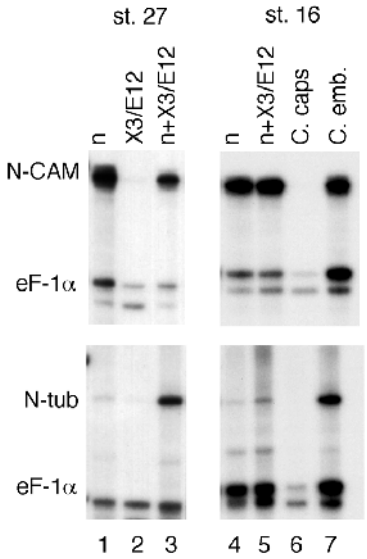

Fig. 2. The ability of *XASH3* to induce the expression of *N-tubulin* in *noggin* caps is delayed to tadpole stages. *Xenopus* embryos were injected with *noggin* RNA, *XASH3* and *E12* RNA or a mixture of *noggin*, *XASH3* and *E12* RNA. Because *XASH3* is more effective at promoting neurogenesis when co-injected with *E12*, a promiscuous bHLH heterodimer partner (Ferreiro et al., 1994), *E12* was included in the intitial experiments. However, *E12* was found not to affect the activity of *XASH3* in *noggin* caps and was therefore omitted from subsequent experiments. Animal caps were dissected at blastula stage, cultured either until the tadpole stage (stage 27, lanes 1, 2, 3) or the neural plate stage (stage 16, lanes 4, 5, 6, 7) and analysed for the expression of the general neural marker *N-CAM*, the neuronal differentiation marker *N-tubulin* and the ubiquitous *eF1-a*. *Noggin* animal caps express only *N-CAM* either at stage 27 (lane 1) or at stage 16 (lane 4). Injection of *XASH3/E12* alone has no effect (lane 2), but co-injection of *noggin* with *XASH3/E12* RNA activates the expression of *N-tubulin* in addition to *N-CAM*, in stage 27 animal caps (lane 3). However, when *noggin* plus *XASH3/E12*-injected animal caps are analysed earlier, at stage 16, there is no *N-tubulin* expression in either *noggin* or *noggin* plus *XASH3/E12*-injected animal caps (lanes 4 and 5), despite the fact that in control uninjected embryos there is abundant *N-tubulin* expression at this stage (lane 7). Control animal caps dissected from uninjected embryos (C. caps) do not show either *N-CAM* or *N-tubulin* expression (lane 6).

and transcribing with T7 polymerase, as described in Lamb et al. (1993) and the probe for *N-tubulin* is described in Chitnis et al. (1995). The antisense probe for *XBF-1* was prepared from PCR cDNA 210 bp subclone in pKS $^{+}$ by linearising with *Xba*I and transcibing with T3 polymerase. In vitro transcription was also performed as in Harland (1991). *Xotx2/N-tubulin* double in situ hybridisation was performed according to a protocol developed by Doniach and described in Knecht et al. (1995). The samples were first stained with magenta phos and then with BCIP alone. Some specimens were sectioned after staining, and these were fixed O/N in MEMFA, then dehydrated in methanol, permealised briefly (2× 10 minutes in Xylene), followed by 2× 20 minute changes in 1:1 xylene: paraffin wax at 60°C, and embedded in Parafin wax. Sections (10 μm) were cut, dried, dewaxed according to standard histological procedures, mounted in Permount and photographed with Nomarski optics.

RNAse protections

RNA was isolated and assayed by RNAse protections, using P^{32}-labelled antisense RNA probes, as previously described (Melton et al., 1984; Kintner and Melton, 1987). The probes used to detect *N-tubulin*, *N-CAM* and *EF-1α* RNAs have been described previously (Coffman et al., 1990; Ferreiro et al., 1994). To make an antisense probe for *Xotx2*, a 250 bp *Not*I-*Eco*RV fragment from the cDNA clone described in Lamb et al. (1993), pXOT30, was subcloned in pKS^{+}, linearised with *Not*I and transcribed with T7 polymerase. The probe for *XBF-1* was the same as the one used for in situ hybridisation (see above) and the probe for *Hoxb-3* was produced by linearising, with *Xba*I, a *Pst*I-*Xho*I PCR cDNA of approx. 180 bp cloned in pKS^{+} and transcribing with T3 polymerase. Most assays were carried out by assaying RNA samples simultaneously with several probes. In parallel with these multiple hybridisation reactions, control embryo RNA was hybridised separately with each probe in order to show the expected size of the protected fragment for each probe. RNA isolated from 5 to 10 animal caps was analysed in each reaction.

PCR cloning of *XBF-1* and *Hoxb-3*

Hoxb-3 was cloned by PCR from a stage 17 cDNA library by using primers against the homedomain of mouse *Hox 2.7* gene. The upstream primer was 5′ TAC ACC TCC GCC CA(GC) CTG GTG GA 3′, corresponding to aminoacid (aa) sequence YTSAQLVE, and the complement of the 3′ primer was 5′ TA(CT) AAG AAG GAC CAG AAG GCC AAG 3′, corresponding to aa sequence YKKDQKAK. *Hoxb-3* was 95% identical to *Hox 2.7* over the amplified region. *XBF-1* was similarly cloned by PCR with primers

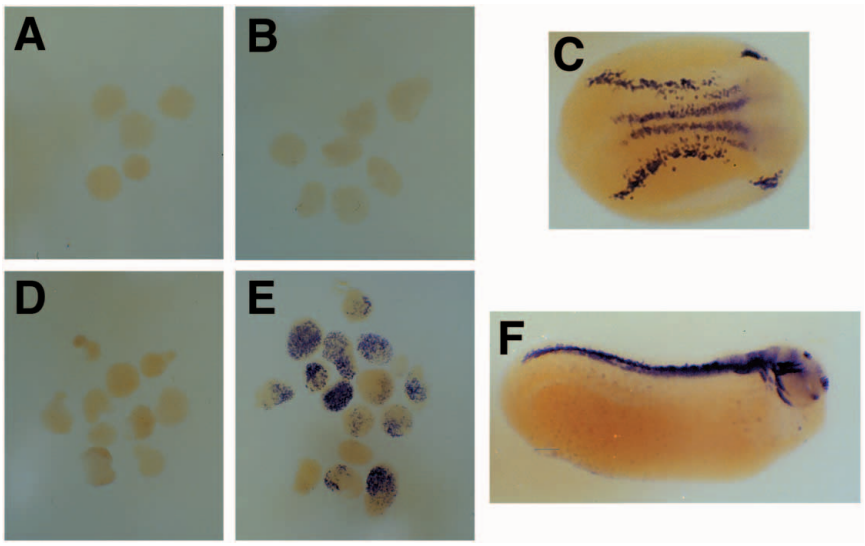

Fig. 3. Whole-mount in situ hybridisation showing that *XASH3* promotes neuronal differentiation in *noggin* caps, but not until the tadpole stage. *Xenopus* embryos were injected bilaterally at the 2-cell stage, with *noggin* (A,D) or *noggin* plus *XASH3* RNA (B,E), animal caps were dissected at blastula stage, cultured either until the neural plate stage (stage 16; A,B) or the tadpole stage (stage 27; D,E) and hybridised with a probe for *N-tubulin*. (C,F) Control embryos at stage 16 and stage 27, respectively. Note that *noggin* alone does not promote the formation of *N-tubulin*-expressing cells either at stage 16 (A) or at stage 27 (D). *Noggin* plus *XASH3*-injected animal caps undergo neuronal differentiation, but only if cultured until stage 27 (E), not at stage 16 (B). In contrast, *N-tubulin* is expressed abundantly at stage 16 (C) in the embryo, suggesting that *noggin* plus *XASH3*-injected animal caps need additional signals in order for neuronal differentiation to occur at the neural plate stage.

against the DNA-binding domain of group B of HNF-related proteins (Clevidence et al., 1993) This group includes the rat *BF-1* and the *Drosophila* genes of the *sloppy paired* locus. The upstream primers was 5′ ATG ATG GCN AT(TCA) AG(AG) CA(AG) AG(TC) CCN GA 3′, corresponding to aa sequence MMAIRQSPE and the downstream primer was 5′ GA NGG (AG)TC NAG GAT CCA (AG)TA (AG)TT 3′ the complement of which corresponds to aa sequence NYWMLDPS.

RESULTS

Timing of *N-tubulin* expression differs along the A-P axis of the neural plate of the *Xenopus* embryos

To gain insight into the timing of neurogenesis in vivo, we examined *N-tubulin* expression in neurula and tadpole stage embryos (Fig. 1). When embryos were examined at the neural plate stage, between stages 14 and 16, cells expressing *N-tubulin* were localised to three longitudinal domains on either side of the dorsal midline, which correspond to the three classes of primary neurons that differentiate in the posterior neural tube (Hartenstein, 1989; Chitnis et al., 1995; Fig. 1A). Within these longitudinal domains, *N-tubulin* expression is detected in scattered cells amid a non-expressing population (Chitnis et al., 1995). At the neural plate stage, a second site of expression was also detected in the neurons of the trigeminal ganglion, approximately at the level of *En-2*, an early marker of the midbrain-hindbrain boundary (Fig. 1A, arrow). In contrast to the posterior neural plate, expression of *N-tubulin* at a level anterior to *En-2*, did not appear until after neural tube closure (Fig. 1B, C). *N-tubulin*-positive cells in the forebrain were first detected at the tailbud stage, around stage 25, in the ventral diencephalon, the olfactory placodes and the epiphysis (Fig. 1D). In the next few stages, neurogenesis in the forebrain gradually spread and by the tadpole stage (stage 31), *N-tubulin* was very abundant anteriorly (Fig. 1E) as well as posteriorly (Fig. 1F). Thus, the temporal order of neurogenesis differed in anterior and posterior regions of the neural plate and can be considered to be an early feature of A-P patterning of the neural plate.

Noggin plus *XASH3*-injected animal caps express neuronal markers at neurula but not neural plate stages

To examine the factors that control neurogenesis along the A-P axis of the neural plate, we first examined the timing of *N-tubulin* expression in animal caps that were induced to form neural tissue with *noggin.* From previous studies, we knew that neuronal differentiation is not observed in *noggin* caps at significant levels at least through stage 27 (tadpole stages) (Lamb et al., 1993; Knecht et al., 1995) but that *N-tubulin* expression can be activated in *noggin* caps at these late stages by expressing a *Xenopus achaete-scute* homologue, *XASH3* (Zimmerman et al., 1993; Ferreiro et al., 1994). These observations suggested that *noggin* caps form neuroepithelium that is competent to undergo neuronal differentiation, but it does not do so unless proneural gene activity is increased. To extend on this result, we asked whether the timing of *N-tubulin* expression that can be evoked in *noggin* caps by *XASH3* follows the timing of *N-tubulin* expression observed in embryos.

Animal caps injected with *XASH3* plus *noggin* were isolated, cultured either to the equivalent of stage 16 or stage 27 and were analysed by RNAse protection or in situ hybridisation for *N-tubulin* expression (Figs 2, 3). Consistent with previous reports, at stage 27, *noggin* caps expressed the pan-neural marker *N-CAM* but very low levels of *N-tubulin*; (Fig. 2, lane 1; Fig. 3D; Lamb et al., 1993); this expression was highly enhanced by co-injection of *XASH3* (Fig. 2, lane 3; Fig. 3E; Ferreiro et al., 1994). Apparently, when *noggin* animal caps are cultured longer, other markers of neuronal differentiation such as *sybII* are expressed, but in a pattern that is diffuse (Knecht et al., 1995). In contrast, the expression of *N-tubulin* that was induced by the combination of *noggin* plus *XASH3* at stage 27 appeared punctate (Fig. 3E), reminiscent of the scattered pattern of neuronal differentiation that is observed within the longitudinal stripes in the embryo. Unexpectedly, we found that when assayed at an early time point, i.e. at neurula stage 16, animal caps injected either with *noggin* or *noggin* plus *XASH3* expressed *N-CAM* but not *N-tubulin* (Fig. 2, lanes 4, 5; Fig. 3A,B). This was in contrast with the situation in the embryo where *N-tubulin* was expressed in high amounts by stage 16 (Fig. 2, lane 7; Fig. 3C). These results revealed that, although *XASH3* promotes neurogenesis in *noggin*-injected animal caps, its ability to do so is temporally constrained.

One reason why XASH3 might fail to illicit early *N-tubulin* expression in noggin caps is by promoting a process, called lateral inhibition (Chitnis et al., 1995; Henrique et al., 1995). During normal development, lateral inhibition is thought to limit the number of neurons that form during primary neurogenesis, via local cell-cell interactions that are mediated by a transmembrane receptor, X-Notch-1, and its putative ligand, X-Delta-1. Indeed, blocking *X-Delta-1* function using an antimorphic form of *X-Delta-1*, called *X-Delta-1^{stu}*, has been shown to increase the density of *N-tubulin*-positive cells that form in the posterior neural plate (Chitnis et al., 1995). Furthermore, recent experiments have shown that proneural genes such as *XASH3* increase the expression of *X-Delta-1* and its receptor *X-Notch-1* RNA in the embryo or in *noggin* caps (Turner and Weintraub, 1994; Chitnis and Kintner, 1996), and in high doses inhibit neurogenesis in the embryo (Chitnis and Kintner, 1996). Therefore, it was possible that the delay in neurogenesis in *noggin* plus *XASH3* animal cap was due to excessive lateral inhibition. To test this possibility, we co-injected *X-Delta-1^{stu}* with *noggin* plus *XASH3* to block the effects of lateral inhibition. The result of this experiment (Fig. 4) showed that co-injection of *X-Delta-1^{stu}* with *noggin* plus *XASH3* did not differ from the injection of *noggin* alone (Fig. 4A) or *noggin* plus *XASH3* (not shown), since it did not lead to the formation of *N-tubulin* positive cells in animal caps at the neural plate stage (Fig. 4B), even though the same RNAs produced extensive *N-tubulin* expression in embryos (Fig. 4E), as described previously (Chitnis and Kintner, 1996). Thus, neurons cannot be made to appear earlier by lowering inhibition mediated by *X-Delta-1*. These findings led us to consider other signals in addition to *XASH3*, that are required in order for neurogenesis to take place early in *noggin* caps, synchronously with that seen in vivo.

Retinoic acid induces early *N-tubulin* expression

Based on the observation that neuronal differentiation was delayed in the anterior neuroectoderm (see above), we hypothesised that the delay in neuronal differentiation in *noggin* caps reflected their anterior character. In order to test this hypothe-

sis, we asked whether the timing of *N-tubulin* expression could be altered by an agent that would posteriorise the neural ectoderm. To do this, we examined the effects of retinoic acid (RA), given its known ability to suppress anterior and enhance posterior development in vertebrate embryos (reviewed in Maden and Holder; see also Simeone et al., 1995).

Animal caps expressing *noggin* alone or *noggin* plus *XASH3* were isolated and treated with RA at blastula stage, stage 8-9 (Fig. 5). After the caps were cultured to the equivalent of stage 16, they were assayed for the expression of *N-CAM* and *N-tubulin*. At the neural plate stage (stage 16), *noggin* and *noggin* plus *XASH3*-injected animal caps expressed *N-CAM* but neither one expressed *N-tubulin* (Fig. 5, lanes 1 and 3). In contrast, when *noggin* plus *XASH3*-injected animal caps were treated with RA, we observed a high level of *N-tubulin* expression at stage 16 (Fig. 5, lane 4), which was comparable to the level of *N-tubulin* expression in the embryo at this stage (Fig. 5, lane 5). RA also induced some early *N-tubulin* expression in animal caps injected with *noggin* alone (Fig. 5, lane 2; Fig. 4C); however, this effect was very weak compared to the induction obtained when *XASH3* (Fig. 5, lane 4) or *XASH3* and *X-Delta-1*^{stu} were also present (Fig. 4D). This suggested that RA can induce early neuronal differentiation in *noggin* caps, but that it only does so efficiently when the proneural gene, *XASH3*, is also expressed.

RA posteriorises *noggin*-induced ectoderm

We hypothesised that RA changes the timing of neurogenesis in *noggin* plus *XASH3* caps by changing the positional character from anterior to posterior. To test this, we first examined the regional character of *noggin* plus *XASH3* neuroepithelium. As anterior markers, we used *Xotx2* (Lamb et al., 1993) which is widely expressed across the anterior neural plate and *XBF-1*, the *Xenopus* homologue of the mammalian winged-helix gene *BF-1* (Tao and Lai, 1992). *XBF-1* was expressed in a stripe across the anterior neural plate (Fig. 6) which, according to the Eagleson and Harris (1989) fate map of the neural plate gives rise to the telencephalon. Indeed, in the tadpole, *XBF-1* was expressed specifically in the telencephalon (Fig. 6). As a posterior marker, we assayed for *Hoxb-3* (previously called *Xhox 2.7*), which is expressed in the hindbrain and at lower levels in the spinal cord, as described previously (Dekker et al., 1992; Godsave et al., 1994).

In agreement with previous reports, neural tissue induced by *noggin* alone expressed the anterior marker, *Xotx2* (Lamb et al., 1993; Knecht et al., 1995), the telencephalic marker *XBF-1*, but not the posterior marker, *Hoxb-3* (Fig. 7A, lane 4). Animal caps co-injected with *noggin* plus *XASH3* continued to show expression of *Xotx2* and to lack expression of *Hoxb-3* (Fig. 7A, lane 5). An observed decrease in the levels of the telencephalic marker *XBF-1* in response to *XASH3* could be due to *XASH3* promoting diencephalic differentiation. In sum, these results suggested that the positional character of the *noggin*-induced neural ectoderm was anterior and the presence of *XASH3* did not significantly alter it. When *noggin* and *noggin* plus *XASH3*-injected animal caps were treated with RA at late blastula stage (stage 9), anterior markers such as *Xotx2* and *XBF-1* were down-regulated while the posterior marker *Hoxb-3* was upregulated (Fig. 7A, lanes 1 and 2). Thus, RA did indeed change the positional character of a *noggin* cap from anterior to posterior and at the same time, in co-operation with *XASH3*, it induced early neurogenesis.

To determine whether RA had similar effects in vivo, blastula stage (stage 9) embryos were treated with RA and at neurula stage they were assayed for the expression of *N-tubulin* and *Xotx2* (Fig. 8). In contrast to control embryos (Fig. 8A,D), embryos that were treated with RA showed extensive anterior *N-tubulin* expression (Fig. 8B,E). These embryos also showed a complete loss of *Xotx2* expression, suggesting that anterior development had been suppressed by RA.

RA posteriorises *noggin* caps in the absence of mesoderm

To test whether RA induces posterior mesoderm in *noggin*-injected animal caps which would in turn induce posterior neural tissue and early neuronal differentiation, we examined whether RA induced the expression of *X-bra*, an early mesodermal marker (Smith et al., 1991; Fig. 7B). We found that animal caps injected with *noggin* or *noggin* plus *XASH3* (Fig. 7B, lanes 1 and 2) or caps additionally treated with RA (Fig. 7B, lanes 4 and 5) did not express detectable levels of *X-bra* at the mid-gastrula stage (stage 11), supporting the view that RA can affect the ectoderm directly (Durston et al., 1989; Sive et al., 1990; Sive and Cheng, 1991).

Posterior transformation by RA is coincident with the induction of early *N-tubulin* in *noggin* caps or anterior *N-tubulin* in embryos

The results described above indicated that RA could both posteriorise, and induce early *N-tubulin* expression in *noggin* caps. To examine whether these two effects were linked, we treated *noggin* caps with RA at the end of gastrulation, when the ability of RA to suppress anterior development in embryos is known to decline (Durston et al., 1989; Sive et al., 1990). Indeed, when *noggin* or *noggin* plus *XASH3* caps were treated with RA at the late gastrula stage (stage 12.5; Fig. 7A, lanes 7, 8), the effects on the expression of both anterior and posterior markers were much less pronounced than similar treatment at blastula stage (stage 9; Fig. 7A, lanes 1, 2). As RA lost the ability to completely suppress an anterior fate at stage 12.5, it also lost the ability to induce early *N-tubulin* expression (Fig. 7A, lanes 7, 8 compared to lanes 1, 2).

The same result was obtained when the effect of RA was analysed in whole embryos (Fig. 8). Embryos that were treated with RA towards the end of gastrulation, at stage 12.5, retained some *Xotx2* expression and showed very little *N-tubulin* expression anteriorly (Fig. 8C, F;. see also Ruiz i Altaba and Jessell, 1991) in contrast to embryos that were treated at the blastula stage (Fig. 8B,E). Application of RA at stage 20, i.e. closer to the normal onset of neuronal differentiation in the forebrain, had a small effect on *Xotx2* and no effect on *N-tubulin* expression at stage 27 (data not shown). These results showed that significant anterior *N-tubulin* expression was only detected in embryos in which loss of *Xotx2* had occurred, suggesting that RA could induce early anterior expression of *N-tubulin* only by converting it to posterior tissue.

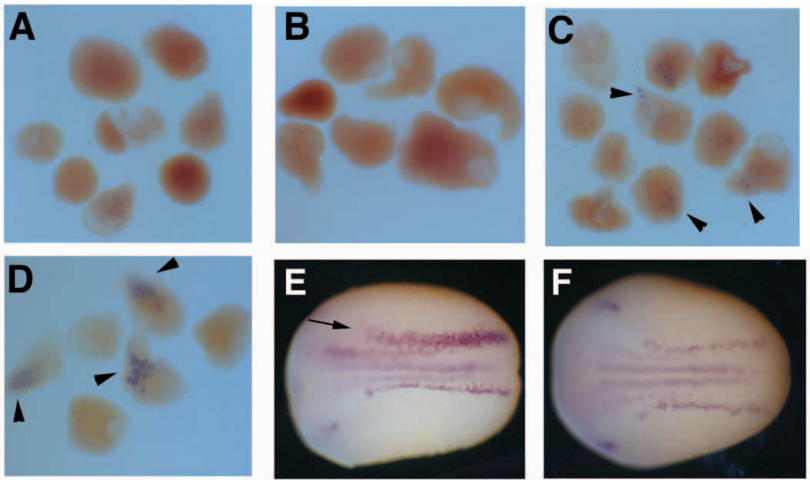

Fig. 4. Blocking lateral inhibition by *X-Delta-1*^{stu} does not promote early neurogenesis in response to *XASH3*. Animal caps expressing *noggin* (A) or *noggin* and *XASH3/X-Delta-1* ^{stu} (B) do not express *N-tubulin* at the neural plate stage. In contrast, when animal caps that express *noggin* (C) or *noggin* and *XASH3/X-Delta-1*^{stu} (D), are treated with RA at stage 9 and analysed at the same way as in A and B, they express *N-tubulin*. Highest levels of *N-tubulin* is seen in those co-injected with *noggin* and *XASH3 /X-Delta-1*^{stu} and treated with RA (D). (C,D) Examples of sites of *N-tubulin* hybridisation are shown by arrowheads. In the whole embryo (E), injection of *XASH3/X-Delta-1*^{stu} increases the width and density of *N-tubulin* cells, as previously described (Chitnis and Kintner, 1996). (E) The injected side is shown with an arrow. C, is a control embryo.

DISCUSSION

A principal finding of our studies is that, in *Xenopus* embryos, the neural plate is divided into an anterior and a posterior domain that differ in their schedule of neuronal differentiation. In the anterior domain, which includes the prospective forebrain and midbrain, neuronal differentiation does not appear to occur until closure of the neural tube while, in the posterior domain, which includes the hindbrain and spinal cord, primary neurons are detected in the neural plate shortly after gastrulation. Our results also indicate that neuronal differentiation occurs with a delayed time course in neural tissue induced by noggin in vitro. Thus, the earliest expression of *N-tubulin* in *noggin* caps, even in the presence of *XASH3*, does not occur until tadpole stages, when the anterior domain in the embryo normally expresses *N-tubulin*. These results show that *XASH3* induces neuronal differentiation in neuralised ectoderm, but its ability to do so is temporally constrained. These observations led us to hypothesize that this temporal constraint on neuronal differentiation reflects the anterior nature of neural tissue induced by noggin, and to predict that this timing could be changed from late to early by supplying signals that changed the noggin-induced neural tissue from anterior to posterior. Indeed, treating *noggin* caps with the posteriorizing agent, RA, suppresses anterior neural markers, induces posterior neural markers, and changes the timing of neuronal differentiation to an earlier, neural plate stage. These results, therefore, suggest that the timing of neuronal differentiation is linked to the process of patterning the neural plate along the A-P axis during neural induction.

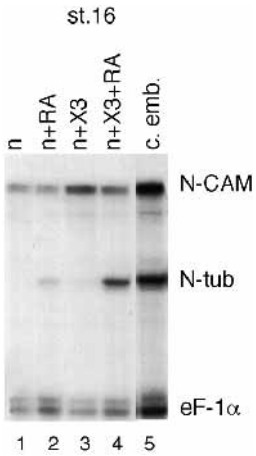

Fig. 5. RA co-operates with *noggin* and *XASH3* to induce *N-tubulin* expression at the neural plate stage. Animal caps expressing *noggin* (n; lane 1) or *noggin* plus *XASH3* RNA (n+X3; lane 3), were dissected at blastula stage, half were treated with RA (n+RA, lane 2 and n+X3+RA, lane 4) and all were analysed by RNAse protection at the neural late stage (stage 16) for the expression of *N-CAM* and *N-tubulin*. Note that, while all the *noggin* caps have been neuralised and express *N-CAM*, only those that have been co-injected with *XASH3* and treated with retinoic acid express significant amounts of the neuronal differentiation marker *N-tubulin* (lane 4). The level of *N-tubulin* that is induced by the *noggin*+*X3*+RA (lane 4) combination is comparable to the levels of *N-tubulin* expression in control embryos (c. emb.; lane 5) of the same stage (stage 16).

Regulation of neuronal differentiation in anterior neuroectoderm

Why is the *noggin*-induced anterior neuroectoderm or the anterior neural plate, refractory to neuronal differentiation at early stages? The fact that this block cannot be overcome by *XASH3* suggests that the anterior neuroectoderm contains inhibitors of proneural gene activity. At least three types of inhibition are known to affect that activity of proneural genes. One type is a form of local cell-cell interaction mediated by the inhibitory ligand *X-Delta-1* and its receptor *X-Notch-1* in a process known as lateral inhibition. Initially, it seemed likely that lateral inhibition contributes to the delay in neuronal differentiation seen in *noggin* caps because expression of

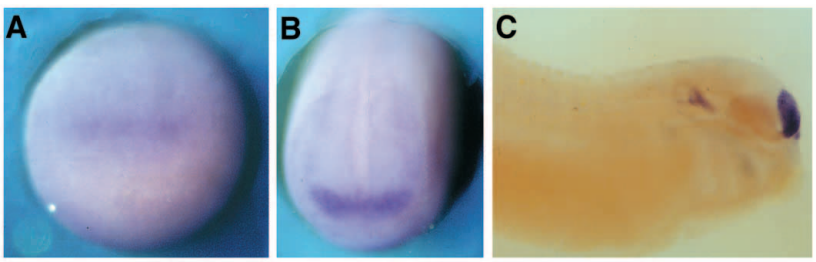

Fig. 6. *XBF-1* expression marks the developing telencephalon in *Xenopus* embryos. Whole-mount in situ hybridisation shows that *XBF-1* is expressed at the anterior end of the neural plate at the late gastrula (stage 12.5 ; A) and the neural plate stages (stage 16; B). At the tadpole stage (stage 27), *XBF-1* is expressed in neuroepithelial cells of the telencephalon (C).

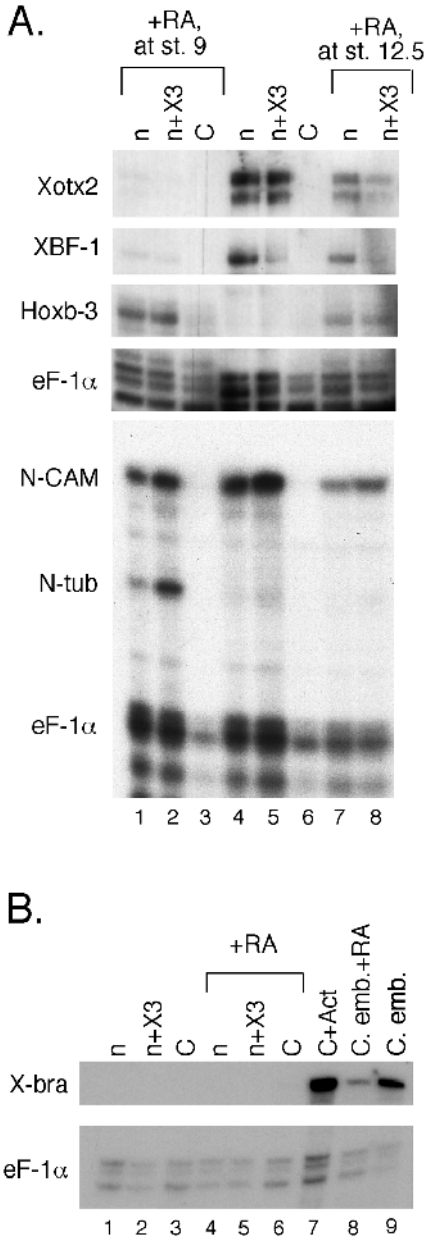

Fig. 7. (A) RA applied at blastula stages but not at late gastrula stages posteriorises *noggin* caps and simultaneously induces *N-tubulin* expression. *Xenopus* embryos were injected at the 2-cell stage with *noggin* RNA (n; lanes 1, 4, 7) or *noggin* plus *XASH3* RNA (n+X3; lanes 2, 5, 8). Animal caps were dissected at the blastula stage (stage 8), and either treated with RA when sibling embryos reached blastula (stage 9; lanes 1, 2) or late gastrula stage (stage 12. 5; lanes 7, 8), or left untreated (lanes 4, 5). RNA from these three groups was isolated at the neural plate stage (stage 16) and was divided into two parts, one analysed simultaneously for the expression of *Xotx2*, *XBF-1*, *Hoxb-3* and *eF1-a* and the other analysed simultaneously for the expression of *N-CAM*, *N-tubulin* and *eF-1a*. Note that *noggin* (lane 4) and *noggin* plus *XASH3* (lane 5)-injected animal caps express the anterior markers *Xotx2* and *XBF-1*, but not the posterior marker *Hoxb-3*. These animal caps express *N-CAM* but no *N-tubulin*. In animal caps that have been similarly injected and dissected but then treated with RA at stage 9 (lanes 1, 2), the anterior markers *Xotx2* and *XBF-1* are suppressed and the posterior marker *Hoxb-3* is induced. The same animal caps show both *N-CAM* and *N-tubulin* expression; however, the level of *N-tubulin* is significant higher when both *XASH3* and RA are present (lane 2). In animal caps that have been similarly injected and dissected but then treated with RA at late gastrula (stage 12.5; lanes 7, 8) instead of blastula stage (stage 9; lanes 1, 2), the anterior markers *Xotx2* and *XBF-1* are suppressed and the posterior marker *Hoxb-3* is induced but to a lesser degree than in animal caps treated with RA at blastula stage (stage 9). These animal caps express *N-CAM* but no *N-tubulin*. (lanes 7, 8). Animal caps that were dissected from uninjected embryos (control caps; C) do not express any of the regional or neural markers either with or without RA treatment (lanes 3, 6), demonstrating that RA alone is not a neural inducer. (B) RA does not induce *X-bra* expressing mesoderm in *noggin* or *noggin* plus *XASH3*-injected animal caps. Animal caps expressing *noggin* RNA (n) or *noggin* plus *XASH3* RNA (n+X3) were dissected at the blastula stage (stage 8), treated with RA when sibling embryos reached stage 9, harvested for RNA isolation at stage 11 and analysed for the expression of the early mesodermal marker *X-bra*, which is normally expressed in embryos at this stage (C. emb., lane 9). Neither *noggin* (lane 1) nor *noggin* plus *XASH3* (lane 2)-injected animal caps expressed *X-bra* and RA did not induce its expression (lanes 4, 5). As a positive control, animal caps were dissected from uninjected embryos and were treated with activin. These were also analysed at stage 11 and, as expected, were found to express high levels of *X-bra* (lane 7). In the whole embryo, RA suppressed the expression of *X-bra* (C. emb. +RA; lane 8). Control animal caps were isolated from uninjected embryos (C; lanes 3, 6).

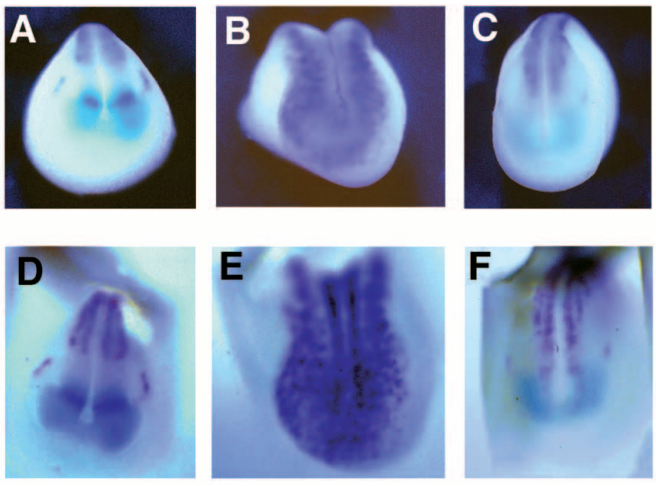

Fig. 8. RA applied at blastula stages but not at late gastrula stages, posteriorises the anterior neural plate and simultaneously induces premature anterior *N-tubulin expression*. *Xenopus* embryos were either left untreated (A,D) or treated with RA when sibling embryos reached stage 9 (B,E) or stage 12. 5 (C,F), and analysed simultaneously for the expression of *Xotx2* and *N-tubulin*. *Xotx2* staining is light blue while *N-tubulin* staining is magenta in color. Embryos shown in A-C have been cleared in benzyl benzoate in order to reveal possible *N-tubulin* staining in the deep regions of the neural tissue and are shown in D-F, respectively. Uninjected embryos expresses *Xotx2* but no *N-tubulin* in the anterior neural plate (A,D). In embryos that have been treated with RA at stage 9, the anterior markers *Xotx2* is suppressed and *N-tubulin* is massively induced in the anterior end of the neural ectoderm (B,E). In embryos that have been treated with RA at stage 12.5 the anterior marker *Xotx2* is suppressed to a lesser degree than in embryos treated with RA at stage 9 (C,F). When cleared, these embryos show few *N-tubulin* cells anteriorly (F).

XASH3 either in *noggin* caps or whole embryos activates the expression of the inhibitory ligand *X-Delta-1*. This in turn inhibits the ability of *XASH3* to promote neurogenesis in the embryo (Chitnis and Kintner, 1996). However, our results have excluded this possibility by showing that lowering the activity of *X-Delta-1*-with an *X-Delta-1*-antimorph, still did not allow *XASH3* to promote early neuronal differentiation in a *noggin* cap. A second potential source of inhibition reflects the fact that bHLH transcription factors, such as *XASH3*, operate as heterodimers and, indeed, are subjected to negative control when they form inactive dimers with HLH proteins, called IDs, that lack basic DNA-binding domains (e. g. Garrell and Modolell 1990; Van Doren et al., 1991; Cabrera et al. 1994). At present, it is not known whether ID-like proteins are expressed in *Xenopus* embryos and whether they are active in the anterior neural plate. Finally, a third type of inhibition is mediated by bHLH transcription factors that bear a characteristic four amino acids, WRPW, at the carboxy terminus, such as the *Hairy* gene in *Drosophila*, which antagonises the formation of neural precursor cells (e. g. Van Doren et al., 1994). A recent knock out of the *Hairy*-related gene in the mouse, called *HES-1*, leads to excessive and premature differentiation of neuronal cells in anterior regions of the neural tube (Ishibashi et al., 1995). At least two *Hairy* related genes have been identified in *Xenopus* which are expressed in the anterior neural plate and *noggin* caps (D. Turner, pers communication). Thus, one possibility is that inhibitors such as the *Hairy* genes, or the *ID*s, are targets of regulation by A-P patterning and this regulation controls the timing of neuronal differentiation. At present, we cannot rule out the possibility that there are unidentified positive cofactors required for neuronal differentiation that are present in the posterior neural plate and absent from the anterior neural plate. We should also emphasize that at present we do not know whether the delay in neurogenesis in the anterior neural plate reflects a delay in the timing of forebrain neuron birth or a delay in the expression of late differentiation markers such as *N-tubulin* by anterior neurons.

Role of RA in regulating neurogenesis

The observation that *noggin* caps express many of the same regional neural markers as the anterior neural plate suggested that the temporal constraint on neuronal differentiation seen in *noggin* caps reflects its anterior character. Our results support this hypothesis by showing that RA can both posteriorise and change the timing of neuronal differentation in both *noggin* caps and in embryos. One interpretation of these results is that posteriorisation of anterior neuroectoderm, and induction of early neuronal differentiation by RA, are two separate events occurring in parallel. However, several lines of evidence support our favored interpretation which is that RA induces early *N-tubulin* expression via its ability to change the expression of region specific genes such as *Xotx2*, *XBF-1* and *Hoxb-3*. First, embryos and animal caps lose the competence to respond to the posteriorising and the neuronal inducing effects of RA at about the same time, suggesting that two effects of RA may be linked, at least in this system. Second, a mouse knock-out of *BF-1*, the mouse homologue of *XBF-1*, shows premature neuronal differentiation in the forebrain, providing a link between region-specific genes and neuronal differentiation (Xuan et al., 1995). Finally, a dominant negative RA receptor has been shown to interfere both with posteriorisation and with neurogenesis in whole embryos (Blumberg et al., under consideration by *Science*). To determine the link between A-P patterning and neuronal differentiation, it will be important to clarify further the molecular mechanisms by which RA exerts its effects.

Regulation of neuronal differentiation by A-P patterning

Recent studies indicate that neural tissue is initially induced in dorsal ectoderm by antagonising ventralising signals, such as BMP-4, through the action of organiser signals such as noggin, follistatin and chordin. However, neuroectoderm which is induced by a neuralising factor alone differs from neuroectoderm that forms in vivo in at least two ways. First, it is not patterned along the A-P axis since it shows only limited anterior characteristics and, second, it does not undergo neuronal differentiation, at least through tadpole stages. Thus, following the initial neuralisation two additional processes must take place.

The first one, patterning along the A-P axis, is likely to involve additional signals generated during neural induction. One view, based on the activation/transformation model of neural induction (reviewed in Doniach, 1993), is that these additional signals will not neuralise ectoderm on their own, but act in combination with neural inducers such as noggin to generate other regions of the nervous system. While recent studies have implicated FGF and wnt-3A in the process of posteriorisation (Cox and Hemmati-Brivanlou, 1995; Lamb and Harland, 1995; Launay et al., 1996; Kengaku and Okamoto, 1995; McGrew et al., 1995), the ability of RA to suppress anterior and promote posterior gene expression in a *noggin* cap in the absence of mesoderm suggests that it acts as a direct posteriorising agent. Since *noggin* caps completely lack expression of genes posterior to the midbrain, RA cannot be acting to elevate pre-existing posterior gene expression but instead is likely to be converting forebrain neural tissue to posterior, thus supporting the idea that RA treatment produces an anterior-to-posterior transformation (Durston et al., 1989; reviewed in Maden and Holder, 1992). Furthermore, while a role for endogenous FGF receptor signalling is unlikely (Kroll and Amaya, 1996), endogenous retinoid receptor signalling appears to have role in A-P patterning in vivo (Blumberg et al., under consideration by *Science*).

The second process, neuronal differentiation, is likely to involve the activation of proneural genes that define domains of neuronal competence within the neural plate (Ferreiro et al., 1994; Chitnis et al., 1995; Chitnis and Kintner, 1996). The signals responsible for increasing proneural gene activity in selected regions of the neural plate are not known. Nonetheless, our findings indicate that the processes that control the activity of the proneural genes and those that control A-P patterning are likely to interact. Specifically, we propose that process of A-P patterning regulates the activity of proneural genes such that their neural promoting effect in the anterior neuroectoderm is delayed until after neural tube closure. We have shown that RA is involved in this process while the effect on neurogenesis of other potential posteriorisers such as FGF-related or Wnt-related signals remains to be explored. An important problem for future research is to determine the

precise molecular mechanisms by which A-P patterning and neurogenesis are integrated during CNS development.

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