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. Concomitantly 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 results 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 bona fide 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 as 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, 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 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 Not1.
and transcribing with T7 polymerase, as described in Lamb et al. (1993) and the probe for N-tubulin is described in Chintis et al. (1995). The antisense probe for XBF-1 was prepared from PCR cDNA 210 bp subclone in pKS+ by linearising with XbaI and transcribing 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, permeabilised briefly (20 min) were cut, dried, dewaxed in xylene, followed by 20 minute changes in 1:1 xylene: paraffin wax at 60°C, and embedded in Paraflin wax. Sections (10 μm) were cut, dried, de waxed according to standard histological procedures, mounted in Permount and photographed with Nomarski optics.

**RNAse protections**

RNA was isolated and assayed by RNase protections, using 32P-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 NotI-EcoRV fragment from the cDNA clone described in Lamb et al. (1993), pXOT30, was subcloned in pKS+, linearised with NotI 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 XbaI, a PstI-XhoI 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(G) CTG GTG GA 3', corresponding to aminoacid (aa) sequence YTSQVLE, and the complement of the 3' primer was 5'TA(C) AAG AAG GAC CAG AAG GCC AAG 3', corresponding to aa sequence YKDDQKAK. Hoxb-3 was 95% identical to Hox 2.7 over the amplified region. XBF-1 was similarly cloned by PCR with primers

**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 initial 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).

**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 (Clevende 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(TG) CCN GA 3', corresponding to aa sequence MMAIRQSPF 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 RNAs 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 coinjection 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 at 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 anti-morphic form of X-Delta-1, called X-Delta-1-sm, 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 caps was due to excessive lateral inhibition. To test this possibility, we co-injected X-Delta-1-sm 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-sm 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 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.
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.

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

![Image](image-url)
Neuronal differentiation in Xenopus neuroectoderm

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-α and the other analysed simultaneously for the expression of N-CAM, N-tubulin and eF-1α. 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 (stage 12.5; lanes 7, 8) instead of blastula stage (stage 9), 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 express 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 IDs, 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 differentiation 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 posteriorize 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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REFERENCES


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