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noggin-Expressing Ectoderm Cells to Activate *N-tubulin* and Become Neurons

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Neurotransmitters regulate neuronal function in the nervous system and modulation of their synthesis, release, and binding by immature neurons and their targets is a major part of nervous system development. We propose that the neurotransmitter noradrenaline regulates neuronal fate during neurulation, before neurons have differentiated. The ability of noradrenaline to induce a neural fate was tested in naive ectoderm caps cut from late blastula stage *Xenopus* embryos. Noradrenaline (10^{-6} M) did not switch on *otx*-2 or NCAM and did not induce the formation of cement glands. We conclude that noradrenaline cannot induce a neural fate. By contrast, 10^{-8} M noradrenaline activated *N-tubulin* in ectoderm caps expressing the neural inducing molecule *noggin* by the time intact siblings had become mid-neurulae. Methoxamine, a specific α-adrenergic receptor agonist, also activated *N-tubulin* in *noggin*-expressing caps. The α-adrenergic receptor blocker prazosin inhibited both noradrenaline- and methoxamine-induced activation of *N-tubulin*. The neurotransmitters dopamine and 5-HT did not activate expression of *N-tubulin*. *XA*-1, *Otx*-2, *X-Delta*, and *Xotch* transcripts were not sensitive to noradrenaline. *Hox*B9, which indicates posteriorization, was not activated by noradrenaline. When intact siblings were at stage 27, many cells in *noggin*-expressing, noradrenaline-treated caps were stained by the neuron-specific mcAb3A10. We propose that noradrenaline is an important endogenous modulator of neuronal fate, driving *noggin*-expressing cells to become neurons by binding to α-adrenergic receptors and activating a cascade that culminates in the expression of the neuronal markers *N-tubulin* and 3A10.

Key Words: **neurotransmitter; *Xenopus; noggin*; embryo; neural induction; noradrenaline; *N-tubulin;* neuron.**

INTRODUCTION

The nervous system arises as a result of an inductive interaction between cells of the dorsal mesoderm and dorsal ectoderm during gastrulation. As a result, dorsal ectoderm cells begin to follow a neural fate. Although the precise mechanisms underlying neural induction have yet to be defined, progress has been made in identifying genes that are activated in dorsal ectoderm cells as a result of neural induction, particularly in *Xenopus*. Thus, *XA*-1 characterizes the cement gland and hatching gland, while *otx*-2 marks the cement gland and the forebrain (see Sive and Bradley, 1996). Activation of mRNAs for the neural cell adhesion molecule NCAM in neuroepithelial cells at the end of gastrulation provides a key indicator of successful neural induction (Kintner and Melton, 1987). This is followed, immediately after gastrulation, by the appearance of mRNAs for genes such as *Xash* -3 (Ferreiro *et al.*, 1994), *Neurogenin* (Ma *et al.*, 1996), and *Xotch* and *X-Delta* (Chitnis *et al.*, 1995). *X-Delta* marks six stripes of neural plate cells on either side of the midline, in positions that correspond approximately to the future sites of differentiation of motor neurons (central), early interneurons (middle), and primitive sensory neurons, the Rohan Beard cells (lateral). mRNAs for *NeuroD* and *N-tubulin* are detected subsequently. Current evidence, based on overexpression or ectopic expression of mRNAs, suggests that *NeuroD* is one of the genes that determines whether cells are neural or epidermal in fate, while *X-Delta* interacts with *Xotch* to control the proportion of neural plate cells that subsequently differentiate into neurons (Chitnis *et al.*, 1995). *Xotch* also may control the competence of cells to respond to inductive signals (Papalopulu and Kintner, 1996). Differentiated

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neurons, which express *N-tubulin*, and glial cells in the early nervous system can be identified reliably by antibodies to neurofilament protein and GFAP about 4 h after neural tube closure (Messenger and Warner, 1989).

The initiating process, neural induction, has been elusive. Smith and Harland (1992) discovered a gene, called *noggin*, which was switched on during gastrulation and displayed many of the properties to be expected from a gene whose product was involved in neural induction. *Noggin* was identified by its ability to restore a dorsal axis to UV-irradiated embryos, which lack dorsal structures. In ectoderm caps dissected from embryos injected with a *noggin*-expressing cDNA construct, NCAM is switched on and cement glands, an early anterior structure, are formed. *Noggin* protein applied directly to ectoderm caps cut from late blastulae has the same effects (Lamb *et al.*, 1993). However, neither *noggin*-expressing nor *noggin*-protein-treated ectoderm caps generate cells expressing *N-tubulin*, a marker of differentiated neurons, until intact siblings are swimming tadpoles.

One possibility is that an activating signal that is present in normal development is not generated when neural induction is achieved by *noggin* alone. Rowe *et al.* (1993) discovered that inhibiting dopamine-β-hydroxylase, the synthetic enzyme for the neurotransmitter noradrenaline, in neurulating embryos prevented the subsequent differentiation of CNS neurons. Furthermore, blockers at α-adrenergic receptors, such as prazosin, applied during neurulation, also inhibited the subsequent differentiation of CNS neurons.

These observations suggest that noradrenaline might be an endogenous signaling molecule, which ensures that neural induction culminates in the generation of differentiated neurons. Neurotransmitters are major regulators of neuronal function in the adult nervous system and modulations of neurotransmitter synthesis, release, and binding by immature neurons and their targets are important while the architecture of the adult nervous system is being established. However, the possibility that neurotransmitter molecules might play a role earlier in nervous system development, before neurons have differentiated, has been little considered. *Xenopus* embryos maintain substantial intracellular stores of dopamine, the immediate synthetic precursor of noradrenaline, in dorsal mesoderm and ectoderm cells (Rowe *et al.*, 1993); Rowe *et al.* further showed that interfering with either noradrenaline synthesis or binding during neurulation inhibited the subsequent differentiation of CNS neurons, making an endogenous role for noradrenaline signaling during neurulation entirely plausible.

In this paper we use naive ectoderm caps cut from *Xenopus* embryos to determine whether noradrenaline might be a primary neural inducing molecule. We then use *noggin*-expressing ectoderm caps to test whether noradrenaline can activate *N-tubulin* and drive *noggin*-expressing cells to become neurons.

MATERIALS AND METHODS

Adult female *Xenopus laevis* were injected with chorionic gonadotrophins to induce egg laying. The following day mature eggs were expressed from the females and fertilized artificially with a macerated testis. Fertilized eggs were stripped of their jelly coats with 2% cysteine (pH 8.0) in Holtfreter's solution and injected with 20 to 40 pg of pCSKa *noggin* cDNA (gift of R. Harland). Ectoderm caps were cut from control and injected embryos when they reached the late blastula stage; transferred to NAM (normal amphibian medium: Slack, 1984) or Holtfreter's solution containing noradrenaline (NA), dopamine (DA), 5-hydroxytryptamine (5-HT), or methoxamine (M), as appropriate; and left until intact siblings had reached the required stage. Glutathione was included along with NA to prevent oxidative breakdown and prazosin, a specific α-adrenergic receptor blocker (Cavero and Roach, 1980), included to test whether noradrenaline was acting through α-adrenergic receptors. When sibling embryos reached the required stage caps were frozen rapidly in minimal fluid.

Analysis of RNAs

Caps were homogenized in buffer (50 mM Tris–HCl, pH 7.5, 1 mM EDTA, 1% SDS, 0.3 M NaCl), extracted twice with phenol/chloroform at room temperature, followed by ethanol precipitation at −20°C to give total RNAs. RNAse protections assays (RPA) were carried out as described by Melton *et al.* (1984). Briefly, probes were transcribed at 37°C for 60 min and template was removed with RNAse-free DNAse. Probes were gel purified and coprecipitated with RNA extracted from 5 to 10 ectoderm caps. Pellets were resuspended in 40 μl hybridization salts (40 mM Pipes, pH 6.4, 1 mM EDTA, pH 8.0, 0.4 M NaCl) with 80% formamide, denatured, hybridized overnight, and digested with RNAse T1. Protein was removed with 50 μg proteinase K in 0.5% SDS. Samples were then phenol/chloroform extracted, reprecipitated in ethanol at −20°C, pelleted, resuspended in buffer, denatured at 80°C for 5 min, and run on a sequencing gel. The following antisense probes were used *XA*-1 (272-nt 5′-end coding region), *Otx2* (200-nt 5′-end coding region), NCAM (219 nt, protected fragment 204 nt; Kreig *et al.*, 1989), *X-Delta* (220-nt fragment from the 3′-end), *Xotch* (213-nt mid coding region), *Hox*B9 (236 nt, 5′ end including homeobox region), and *N-tubulin* (202 nt, bases 234 to 436; Good *et al.*, 1989). In the region of the probe, *N-tubulin* shares only 80% homology with all other tubulins so far sequenced. All samples were assayed simultaneously with an ornithine decarboxylase (ODC) probe (gift of L. Dale, 90-bp protected fragment) to control for RNA loading.

Whole-mount *in situ* hybridization was carried out on intact ectoderm caps according to Harland's (1991) method. Briefly, caps were incubated in 8 μg/ml proteinase K (Sigma) at room temperature for 5 min, prehybridized for 4 h at 60°C in buffer (50% formamide, 5× SSC, 500 μg/ml torula RNA, 100 μg/ml heparin, 1× Denhardt's (Sigma), 0.1% Chaps. 5 mM EDTA), and then hybridized overnight with 500 ng/ml probe in the same buffer. Caps were washed several times at 60°C, blocked with 20% heat-treated lamb serum for 1 h at RT, and then incubated overnight in 1:1000 affinity-purified sheep antidigoxigenin coupled to alkaline phosphatase (Boehringer) with 20% lamb serum to reduce background. Caps were incubated overnight in an NBT/BCIP solution (Boehringer) to reveal staining and fixed in Memfa (0.1 M Mops, pH 7.4, 2 mM EGTA, 1 mM $MgSO_4$, 3.7% formaldehyde) to stabilize the stain. Stained caps were dehydrated rapidly (5 min each) in a series of alcohols and

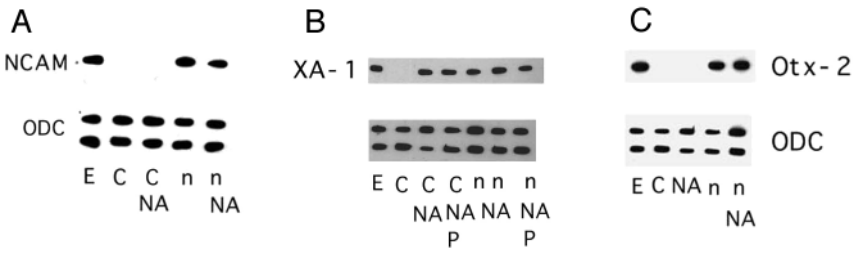

FIG. 1. Noradrenaline is not a primary neural inducer. Ectoderm caps from late blastula stage embryos treated with noradrenaline and assayed at stage 17 (A) or stage 12/13 (B, C). E, intact embryos. C, ectoderm caps. NA, ectoderm caps treated with noradrenaline. n, *noggin*-expressing caps. n/NA, *noggin* caps treated with noradrenaline. (A) Noradrenaline does not induce expression of NCAM in naive ectoderm caps. (B) NA induces *XA*-1, but this is not prevented by prazosin. (C) NA does not induce *Otx*-2.

infiltrated (83% hydroxyethyl methacrylate, 17% butoxyethanol, 0.1% benzoyl peroxide) for 10 min at RT. They were embedded in methacrylate at room temperature overnight, attached to chucks, and sectioned at 5–8 μm on a Sorvall TB-4 Microtome. *N-tubulin* transcripts were detected with an antisense RNA probe (202 nt, bases 234 to 436; Good *et al.*, 1989). Sense probes were used as controls and gave negligible stain. All probes were precipitated with 0.8 M lithium chloride and ethanol at −20°C and stored in hybridization buffer at −20°C until ready for use.

Sections were photographed with Kodak Ectachrome tungsten reversal film. The resultant transparencies were scanned into Adobe Photoshop with an Epson Filmscan 200 or a Polaroid scanner. It proved effectively impossible to achieve equivalent color rendering for all images; the length of exposure and the methacrylate modified the colors detected by both film and scanners and some adjustment from within Photoshop was necessary.

Detection of Neurons

Ectoderm caps were fixed for 2 h at room temperature in MEMFA when sibling embryos reached stage 27/28 and then stored in PBS at 4°C. For sectioning, caps were incubated in 2% sucrose/PBS for 30 min (room temperature), embedded in OCT, and then frozen in OCT on chucks. Fifteen-micrometer sections were cut on a Bright cryostat, placed on gelatinized slides, and stored at −20°C. Sections were incubated in 2% lamb serum in PBS (30 min), washed three times in PBS (10 min each), and then stained with mcAb3A10 (Developmental Biology Hybridoma Bank, University of Indiana) diluted 1:20 in PBS, 2% fetal calf serum, 0.1% azide, 0.1% Triton X-100 (Sigma) overnight. Sections were rinsed several times in PBS (10 min each) and incubated for 2 h in biotinylated sheep anti-mouse IgG (1:100). After several 10-min PBS washes, sections were incubated in FITC streptavidin (1:100 in PBS/10% FCS) overnight, washed again several times in PBS, with a final overnight wash, and mounted in the antifade mountant Citiflour (City University). Intact embryos were prepared in parallel as a positive control. Sections were viewed on a Leica TDS 4D confocal microscope with excitation wavelengths appropriate for FITC. For caps, optical sections were taken at 2-μm intervals and then recombined to give a projection through the thickness of the section. The first and last optical sections were discarded. Single optical sections were taken from the intact embryos. The resultant images were transferred into Adobe Photoshop.

All conclusions are based on at least three separate experiments.

RESULTS

Noradrenaline Is Not a Primary Neural-Inducing Molecule

The ability of NA to induce ectoderm cells to follow a neural fate was tested by treating ectoderm caps dissected from late blastula stage *Xenopus* embryos with 10^{-6} M noradrenaline. When intact siblings reached stage 17, caps were assayed with RNAse protection assay for NCAM transcripts or stained with antibodies specific for NCAM, as a test for successful neural induction. When intact siblings reached stage 20, some control and NA-treated ectoderm caps were dissociated into single cells, cultured overnight, and then examined for differentiated neurons, muscle cells, and other derivatives (see Rowe *et al.*, 1993, for method). At stage 24/25 the incidence of cement glands, a reliable indicator of dorsoanterior induction, was determined in the remainder.

All tests were negative. Noradrenaline-treated caps did not express NCAM transcripts. Figure 1A shows an RNAse protection assay for NCAM in intact embryos at stage 17 and control, NA-exposed control, *noggin*-expressing, and NA-exposed *noggin* ectoderm caps of the same age. Control (C) and NA-treated caps (NA) did not express NCAM, in contrast to intact embryos (E) and *noggin* caps (n and n/NA). NA-treated caps always failed to stain with NCAM specific antibodies; *noggin*-expressing caps always expressed NCAM protein (data not shown). There were no signs of cement gland formation. No neurons differentiated in cultures of noradrenaline-treated caps. A similar result was obtained with *Otx-2*, another early marker, which is expressed in the cement glands and forebrain (e.g., Sive and Bradley, 1996) (Fig. 1C). By stage 12/13 both intact embryos (E) and

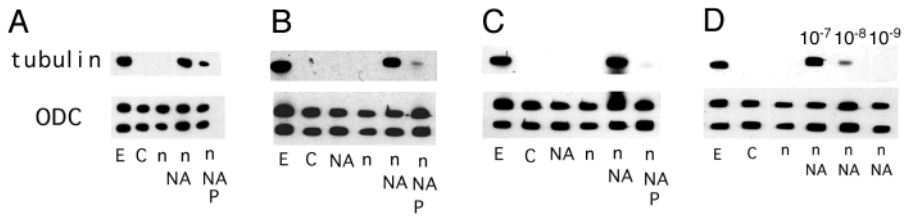

FIG. 2. Noradrenaline induction of *N-tubulin* in *noggin*-expressing caps is prevented by the α-adrenergic receptor blocker prazosin. NA (10^{-6} M): (A) stage 30, (B) stage 17. (C) NA (10^{-7} M), stage 17. (D) Dose–response relationship for induction of *N-tubulin* by noradrenaline. Note that 10^{-8} MNA is sufficient.

noggin-expressing caps (n) expressed *Otx*-2, while ectoderm caps treated with NA did not.

However, NA induced expression of *XA*-1, a marker expressed in the cement and hatching glands, which indicates induction of anterior ectoderm (see Sive and Bradley, 1996). Control ectoderm caps treated with NA and assayed at stage 12/13 expressed *XA*-1 at similar levels to *noggin*-expressing caps (Fig. 1C, NA), although cement glands did not develop subsequently. Prazosin, a specific α-adrenergic receptor blocker, did not inhibit this effect of NA (Fig. 1C, NA/P). We conclude that NA has weak anteriorizing activity.

In *noggin*-expressing caps, NA had no influence on *XA*-1 expression (Fig. 1C, n/NA), *Otx*-2 expression (Fig. 1B, n/NA), NCAM expression (Fig. 1A, n/NA), or the development of cement glands. Ninety-five percent of caps from *noggin*-expressing embryos (approximately 40 pg DNA injected/embryo) had visible cement glands (four experiments: 20 caps/condition/experiment). The same proportion (87%) of *noggin* caps treated with NA developed cement glands. Sixty-five percent of caps from embryos injected with 20 pg *noggin*/embryo developed cement glands whether or not they had been treated with NA. NA had no influence on the frequency with which ectoderm caps treated with *noggin* protein (generated from transfected cell line, gift of R. Harland) developed cement glands (four experiments). We conclude that noradrenaline is not a primary neural-inducing molecule.

Noradrenaline Activates N-tubulin in noggin-Expressing Caps

Ectoderm caps from *Xenopus* late blastulae injected as fertilized eggs with *noggin*-expressing plasmids express NCAM (Fig. 1A and Lamb *et al.*, 1993), implying that expression of *noggin* is sufficient to initiate nervous system development. *Noggin* protein applied to ectoderm caps is equally effective (Lamb *et al.*, 1993). However, *N-tubulin*, which marks neural plate regions destined to give rise to neurons and is expressed subsequently in differentiated neurons, is not activated by *noggin*, at least up to the time when control embryos reach stage 27/28 (Lamb *et al.*, 1993; Ferreiro *et al.*, 1994; Papalopulu and Kintner, 1996).

Noradrenaline promoted expression of *N-tubulin* in *noggin*-expressing ectoderm caps. Figure 2A compares *N-tubulin* mRNAs, assayed by RPA, in intact embryos at stage 30 (E), control caps (C), *noggin* caps (n), and *noggin* caps cultured in 10^{-6} M NA (n/NA) or 10^{-6} M NA + 10^{-6} M prasozin (n/NA/P), an α-adrenergic receptor blocker shown previously to inhibit neuronal differentiation in intact embryos (Rowe *et al.*, 1993). The outcome is clear. Neither control caps (C) nor *noggin* caps (n) express *N-tubulin* at an age when *N-tubulin* is expressed strongly in intact embryos (E). By contrast, *noggin* caps treated with noradrenaline express *N-tubulin* at levels close to that in intact embryos (n/NA). We conclude that by stage 30 NA has induced *noggin* caps to express transcripts for *N-tubulin*. Prasozin suppressed NA-induced *N-tubulin* expression (Fig. 2A, n/NA/P). This implies that NA switches on *N-tubulin* by first binding to α-adrenergic receptors.

In intact embryos *N-tubulin* is switched on during neurulation. Does NA activate *N-tubulin* in *noggin* caps during neurulation? Figures 2B and 2C show that *noggin*-expressing ectoderm caps treated with 10^{-6} (B) or 10^{-7} M (C) NA and assayed when intact siblings reach stage 17 have already activated *N-tubulin*. Furthermore, the specific α-adrenergic receptor blocker prazosin reduced substantially (B) or virtually abolished (C) *N-tubulin* expression. The threshold concentration for induction of *N-tubulin* by NA lies at 10^{-8} M (Fig. 2D).

The ability to induce *N-tubulin* is specific to noradrenaline. Figure 4 compares the consequences of treating *noggin*-expressing caps with the neurotransmitter 5-HT (A) and the neurotransmitter dopamine, the immediate synthetic precursor of NA (B). Dopamine and 5-HT failed to induce expression of *N-tubulin*.

If NA acts by binding to α-adrenergic receptors, it can be predicted that methoxamine, a relatively weak but highly specific α-adrenergic receptor agonist (Starke *et al.*, 1975), should activate *N-tubulin* in *noggin*-expressing caps and that this activation should be inhibited by prazosin. Both of these predictions are correct (Fig. 3C), providing strong support for the view that noradrenaline induces *N-tubulin* expression through activation of α-adrenergic receptors. These results are summarized in Table 1.

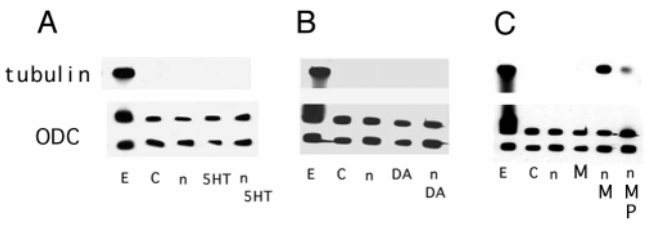

FIG. 3. 5-HT (A) and dopamine (B) do not induce *N- tubulin*. The α-adrenergic agonist methoxamine (M) induces *N-tubulin* and this induction is blocked by prazosin (C).

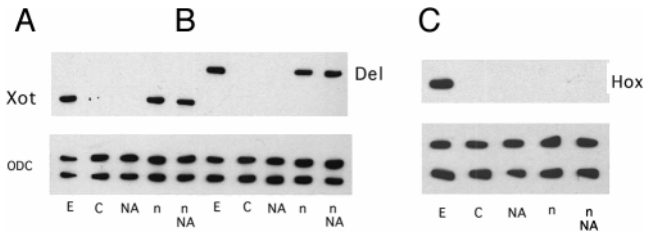

FIG. 4. Noradrenaline does not influence expression of *Xotch* (A), *X-Delta* (B), or *Hox*B9 (C).

Noradrenaline had no influence on the expression of *X-Delta* or *Xotch* (Figs. 4A and 4B and Table 1), implying that altered expression of these genes, both of which are intimately linked with the generation of neurons (Chitnis *et al.*, 1995), does not underlie the activation of *N-tubulin* by noradrenaline.

While these experiments were in progress, Papalopulu and Kintner (1996) reported that retinoic acid induced early *N-tubulin* expression in *noggin* caps and concluded that this was because retinoic acid posteriorized, inducing *Hox*B9 and bringing about *N-tubulin* expression at a time characteristic of posterior structures. *Hox*B9 is expressed in posterior regions of the neural plate and is readily detected at stage 17 (see Fig. 4C, E). Both control (C) and *noggin*-expressing (n) ectoderm caps failed to express *Hox*B9 and noradrenaline did not induce *Hox*B9 (Fig. 4C, NA and n/NA, and Table 1). This makes it unlikely that NA activates *N-tubulin* by posteriorizing *noggin*-expressing caps.

N-tubulin Expression Is Not Uniform

The cellular distribution of *N-tubulin* in noradrenaline-treated, *noggin*-expressing ectoderm caps was determined by *in situ* hybridization. As predicted by the RNAse protection assays, *N-tubulin* was not detected in *noggin* caps (Fig. 5A) when intact siblings had reached stage 17. All NA-treated *noggin*-expressing caps (Figs. 5B and 5C) expressed *N-tubulin*, although not in every cell. *N-tubulin* is excluded from the outer ectoderm. Within the cap interior, many cells express *N-tubulin*, but expression is not uniform. Some cells showed prominent expression, while in others expression is very weak or virtually absent (Fig. 5C).

In the embryo, once the neural tube has closed, *N-tubulin*

TABLE 1

The Consequences of Treatment with Noradrenaline for Gene Expression in Ectoderm Caps

Marker	*XA-1*	*Otx-2*	*NCAM*		*Xotch*	*X-Delta*	*HoxB9*	*N-tubulin*		*3A10*
Developmental stage	12/13	12/13	17	30	17	17	17	17	30	27
Intact siblings	+	+	+	+	+	+	+	+	+	+
Control caps	−	−	−	−	−	−	−	−	−	−
10^{-6} NA	+	−	−	−	−	−	−	−	−	
10^{-6} NA/10^{-6} prazosin	+									
noggin caps	+	+	+	+	+	+	−	−	−	−
10^{-6} NA	+	+	+	+	+	+	−	+	+	+
10^{-6} NA/10^{-6} prazosin	+		+	+				Inhibited	Inhibited	Inhibited
10^{-7} NA								+	+	+
10^{-7} NA/10^{-7} prazosin								Inhibited		
10^{-8} NA								+		
10^{-8} NA/10^{-8} prazosin								Inhibited		
10^{-9} NA								−		
10^{-6} Methoxamine								+		
Methoxamine/prazosin								Inhibited		
10^{-6} Dopamine								−		
10^{-6} 5-HT								−		

Note. +, mRNA detected by RPA. −, mRNA not detected. 3A10, neuron-specific antibody staining. Where no symbol, not determined. All conclusions based on at least three separate experiments.

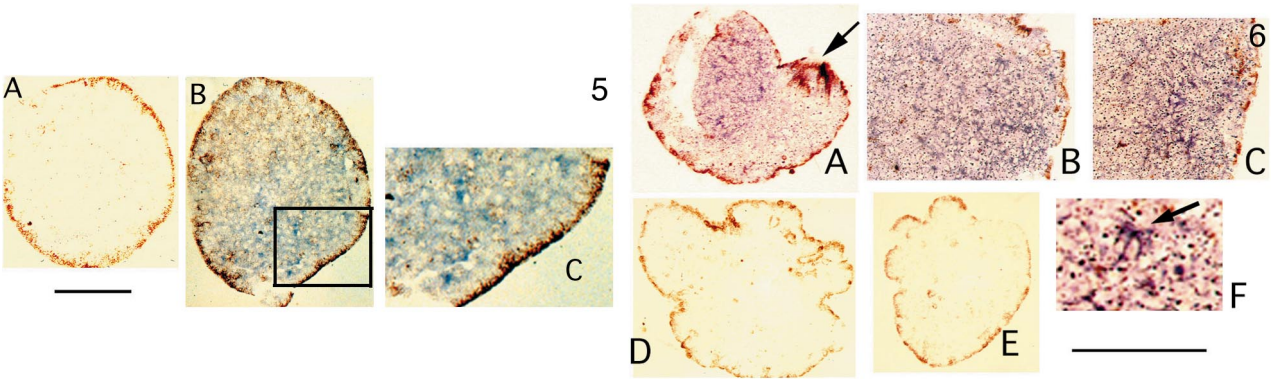

FIG. 5. At stage 17, *N-tubulin*-expressing cells are not uniformly distributed in *noggin*-expressing, noradrenaline-treated ectoderm caps. (A) *noggin*-expressing ectoderm caps do not express *N-tubulin*. (B, C) Noradrenaline (10^{-6} M) induces expression of *N-tubulin*. Cells in the outer layer do not express *N-tubulin* and *N-tubulin*-expressing cells are not distributed uniformly. The high-power image in C shows that *N-tubulin*-expressing cells are interspersed with nonexpressing cells. Bar = 160 μm for A and B and 480 μm for C.

FIG. 6. At stage 27, noradrenaline-treated noggin caps contain many *N-tubulin*-expressing cells. (A–C) *noggin* caps treated with 10^{-6} M noradrenaline. (A) Low-power image to show typical internal organization of Na-treated caps. The *N-tubulin*-expressing cells are concentrated in one region of the cap. The cement gland (arrowed) lies to one side, in a region where the majority of cells do not express *N-tubulin*. (B, C) Sections through two other 10^{-6} M Na-treated *noggin* caps photographed at higher power to show the organization of *N-tubulin*-expressing cells. Note that even in regions where *N-tubulin*-expressing cells are at high density, not all cells express *N-tubulin*. (D) *noggin* cap treated with 10^{-6} M NA and prazosin. This is an example of a cap where prazosin abolished completely the ability of NA to induce *N-tubulin* expression. (E) *noggin* cap that had not been treated with NA. Untreated *noggin* caps never contained *N-tubulin*-positive cells. The scattered dark cells apparent in D and E are pigmented. (F) High-power image to show the stellate shape of *N-tubulin*-expressing cells (arrow) within the noradrenaline-treated cap. Bar = 200 μm for A, D, and E, 125 μm for B and C, and 40 μm for F.

is expressed exclusively in differentiated neurons. Figure 6 shows the distribution of *N-tubulin* in ectoderm caps examined when sibling embryos had reached stage 27. Figures 6A–6C show sections through the *noggin*-expressing cap that had been treated with 10^{-6} M NA. Almost all caps had cement glands (arrowed in the low-power photograph of Fig. 6A). Outer, pigmented cells do not express *N-tubulin*. *N-tubulin*-expressing cells could form both small groups and larger clusters within the interior of the cap. In Fig. 6A, the interior is separated into two regions, one containing a dense array of *N-tubulin*-expressing cells and the other containing larger, flat cells with *N-tubulin* expression in the majority of cells being close to background. In all caps, the cement gland lies in this low-expression region. Figures 6B and 6C show higher-power photographs of *N-tubulin*-expressing regions in two other NA-treated caps. They show that here also not all cells express *N-tubulin*. The characteristic, stellate morphology of *N-tubulin*-expressing cells is illustrated in the high-power image of Fig. 6F. *noggin*-expressing caps treated with NA and the α-adrenergic blocker prazosin (Fig. 6D) failed to express *N-tubulin*, in keeping with the RNase protection assays, where prazosin frequently abolished completely expression of *N-tubulin*. Figure 6E shows an example of the untreated *noggin*-expressing caps, none of which expressed *N-tubulin*.

Noradrenaline Turns noggin-Expressing Cells into Neurons

To demonstrate that noradrenaline not only activates expression of *N-tubulin*, but also switches on neuron specific proteins, we used the monoclonal antibody 3A10 (Serafini *et al.*, 1996). 3A10 has been used previously to mark neurons in chick, mouse, and rat and is an efficient, highly specific marker of neurons in *Xenopus* embryos also (e.g., Fig. 7G). Figure 7 shows that *noggin*-expressing caps treated with NA contained many 3A10-positive cells. Figures 7A–7C show frozen sections through three *noggin*-expressing caps that had been treated with 10^{-6} M noradrenaline. The frozen sectioning procedure introduced a variable degree of damage because of the difficulties associated with complete removal of fluid before transfer to OTC and freezing. This was most apparent at the edge of the caps and there was some fragmentation. Nevertheless, many cells were 3A10 positive. The neurites tended to be very short and tangled. In caps A and C, the neurites were concentrated toward the edge of the region containing the cell bodies (arrowed region). In cap B, short neurites are scattered throughout the cap. The density of 3A10-positive expressing cells varied from cap to cap, but in all cases there were cells that did not stain. These cells often lie together in groups (e.g., top left in B and bottom right in C). In *noggin*-expressing caps treated with 10^{-7} M NA (Fig. 7D), the density of 3A10-positive cells was reduced substantially.

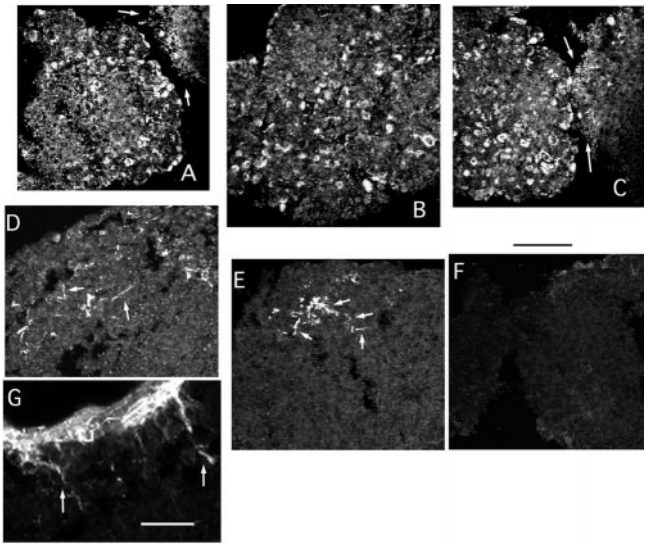

FIG. 7. mcAb3A10 staining reveals that by stage 27 noradrenaline-treated, *noggin*-expressing caps contain neurons. (A, B, C) *noggin* caps treated with 10^{-6} M noradrenaline. 3A10-positive cells are present at high density with many short neurites. In A and C the region containing most neurites lies between the arrows. (D) *noggin* cap treated with 10^{-7} M noradrenaline. The density of 3A10-positive cells is reduced, although neurites are clearly visible (arrow). A substantial proportion of the cap does not contain 3A10-positive cells. (E) Part of *noggin* cap treated with 10^{-6} M noradrenaline and prazosin. The only 3A10-positive cells are present in a small part of the cap (arrows). (F) A *noggin*-expressing cap that had not been treated with noradrenaline. 3A10-positive cells are absent. Each image shows a projection through the frozen section constructed from six stacked 2-μm optical sections. Bar = 100 μm. (G) Neural tube marginal zone in an intact *Xenopus* embryo stained with 3A10. Note dense neurite staining with some neurites coursing into surrounding tissues (e.g., arrows). Single 4-μm optical section. Bar = 50 μm.

Nevertheless, neurites can be detected coursing through the cap. The regions containing no 3A10-positive cells were larger than in caps treated with 10^{-6} M NA. Figure 7E shows that in *noggin*-expressing caps treated with the α-adrenergic blocker prazosin along with 10^{-6} M NA, the incidence of neurons was greatly reduced and neurons were not found in all caps examined (cf. Fig. 6D). None of the *noggin*-expressing, untreated caps contained 3A10-positive cells (e.g., Fig. 7F). Intact embryos at stage 27 cut and stained in parallel with the ectoderm caps showed the characteristic dense stain of neurites in the lateral margins of the mid to hind brain and in the neural tube (e.g., Fig. 7G).

DISCUSSION

Noradrenaline is a potent activator of *N-tubulin* in *noggin*-expressing ectoderm. NA (10^{-8} M) is sufficient to bring about *N-tubulin* expression by the time sibling embryos have reached stage 17. Not every cell in NA-treated *noggin*-expressing caps expressed *N-tubulin* and the cells that expressed *N-tubulin* were not uniformly distributed throughout the cap. The outer, pigmented cells remained *N-tubulin* free. Noradrenaline-induced *N-tubulin* expression is abolished or reduced substantially by prasozin, a specific blocker at α-adrenergic receptors. The specific α-adrenergic agonist methoxamine induced expression of *N-tubulin* and this induction also was inhibited by prasozin. These observations imply that noradrenaline achieves induction of tubulin in *noggin*-expressing ectoderm cells by activating α-adrenergic receptors. When sibling embryos reached stage 27, noradrenaline had not only activated expression of mRNAs for the neuronal marker *N-tubulin*, but also switched on expression of the neurofilament-like protein detected by mcAb 3A10, which is found exclusively in differentiated neurons. This consequence of NA treatment also was substantially inhibited by prazosin.

Dopamine, the immediate synthetic precursor to noradrenaline, is a neurotransmitter in its own right, but was not able to activate *N-tubulin* expression. 5-HT, another well-recognized monoamine neurotransmitter, also was unable to activate *N-tubulin*. These results fit well with those of Rowe *et al.* (1993), who concluded that neither dopamine nor 5-HT was involved directly in controlling neuronal differentiation.

Noradrenaline is not a primary neural-inducing molecule. NA could not activate *Otx*-2 or NCAM in naive ectoderm caps and there were no visible signs of cement glands. However, *XA*-1, a marker of the cement gland and hatching glands, was activated by noradrenaline, suggesting that NA has weak anteriorizing activity. This effect of noradrenaline was not blocked by prazosin, indicating that the weak anteriorizing activity of noradrenaline is not related to its ability to induce *N-tubulin*. Although *Xenopus* embryos maintain intracellular stores of the synthetic precursors of noradrenaline, dopa and dopamine, from fertilization up to closure of the neural tube (Rowe *et al.*, 1993), there is as yet no evidence to indicate whether or not NA plays any part in anteriorizing the embryo during normal development.

By contrast, the observations provide strong support for the view that NA and *noggin* act synergistically to activate the expression of *N-tubulin* during neurulation. Furthermore, they show that NA acts by binding to α-adrenergic receptors, which initiates a cascade that leads to expression of *N-tubulin*. The results further imply that *noggin*-expressing ectoderm cell membranes possess α-adrenergic receptors. However, it is unlikely that *noggin*-expressing cells express the synthetic enzyme dopamine-β-hydroxylase, since the immediate synthetic precursor to noradrenaline, dopamine, was unable to activate *N-tubulin*. This contrasts with the observa-

tions of Rowe *et al.* (1993), who found that dopamine was able to overcome the effects of α-adrenergic receptor inhibition so long as the synthetic enzyme dopamine-β-hydroxylase was available.

NA-treated *noggin*-expressing caps examined by *in situ* hybridization to reveal the distribution of *N-tubulin* expressing cells and stained with the antibody 3A10, a specific neuronal marker in *Xenopus* (present observations) as in other species (Serafini *et al.,* 1996), showed that treatment with noradrenaline not only brought about activation of *N-tubulin* during the neural plate stages, as in normal development, but also provoked the subsequent differentiation of neurons. Neuronal cells tended to form a discreet group within ectoderm caps assayed when siblings had reached stage 27. Thus, noradrenaline not only restores the early expression of *N-tubulin* during neural plate stages, but also imposes the capacity to differentiate into neurons. The finding that not all cells in *noggin*-expressing caps treated with NA express *N-tubulin* or 3A10 is not surprising. NA does not influence the expression of either *X-Delta* or *Xotch* and the normal interaction between cells expressing *X-Delta* and those expressing *Xotch*, which limits the number of neural plate cells that become neurons (Chitnis *et al.,* 1995), can be presumed to be unimpaired.

One possible explanation for the observations would be that noradrenaline mimics retinoic acid, which posteriorizes *noggin*-expressing ectoderm and activates *N-tubulin* (Papalopulu and Kintner, 1996). This seems unlikely. NA does not induce expression of *Hox*B9, a reliable posterior neural plate marker. NA did not reduce the frequency with which either *noggin*-expressing caps or *noggin* protein-treated caps form cement glands, further indicating that NA does not posteriorize. NA does not suppress expression of *XA*-1 or *Otx*-2 in *noggin*-expressing caps, but does induce *XA*-1 in naive ectoderm, which is consistent with weak anteriorizing rather than posteriorizing activity. Therefore, it is unlikely that NA induces *N-tubulin* expression by the same mechanism as retinoic acid. Although we have not carried out exhaustive tests among the many genes activated as a result of neural induction, neither *X-Delta* nor *Xotch*, both of which are closely associated with determining the differentiation of neurons from the neural plate, is influenced by noradrenaline, indicating that the target genes for noradrenaline have yet to be identified.

We conclude that the neurotransmitter noradrenaline is an important promoter of neural development and neuronal fate, acting well before neurons begin to differentiate. Although NA cannot induce neural development in naive ectoderm, it drives induction initiated by *noggin* beyond NCAM, *Xotch*, and *X-Delta* to *N-tubulin* and the generation of 3A10-positive neurons. Prazosin, a specific α-adrenergic receptor blocker,opposes NA- and methoxamine-induced expression of *N-tubulin* and NA-driven expression of 3A10-positive cells, suggesting that NA binds to α-adrenergic receptors. This ability is not shared by other monoamine neurotransmitters, including dopamine, the immediate synthetic precursor to noradrenaline.

The present observations complement our earlier experiments (Rowe *et al.,* 1993). Extracts of intact embryos at stages 12 and 14 contained just detectable levels of noradrenaline. The extracellular space between the dorsal mesoderm and neural plate is narrow and even 10^{-6} M endogenous noradrenaline would be diluted many fold in a whole embryo extract; 10^{-8} M NA was sufficient to induce *N-tubulin* expression. The differentiation of CNS neurons from the neural plate could be suppressed either by preventing synthesis of NA or blocking NA receptors during neurulation. Together our results provide strong evidence for the view that the neurotransmitter noradrenaline is an early, endogenous promoter of neuronal fate and differentiation.

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