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The role of noradrenaline in the differentiation of amphibian embryonic neurons

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SUMMARY

The possibility that monoamines might act as signalling molecules during the early development of the nervous system has been examined in embryos of the amphibian *Xenopus laevis*. The distributions of 5-hydroxytryptamine, dopamine, noradrenaline and their precursor, dopa, were determined from the fertilized egg up to the late neurula stages using High Performance Liquid Chromatography, formaldehyde-induced fluorescence and antibody staining. 5-hydroxytryptamine was not detected until the tail bud stage. The fertilized egg contained significant concentrations of dopa (10^{-6} M) and dopamine (10^{-7} M). Both monoamines persisted with little change in concentration up to the late neurula stage. Early neurula stage embryos contained very low levels of noradrenaline. Aldehyde-induced fluorescence showed that monoamines are localized in dorsal regions of the embryo, in ectoderm and mesoderm cells. Monoamines were not present in endoderm cells. Immunocytochemical staining showed dopamine predominantly in the ectoderm, except in future neural regions where it was found also in the mesoderm. Dopamine staining was always most intense in dorsal regions of the embryo. The consequences for subsequent neuronal differentiation of interfering with the biosynthesis and receptor binding of monoamines during neurulation was assayed. Neuronal differentiation was monitored quantitatively in cultures set up as the neural tube closed and qualitatively in intact tadpoles that were left to develop for two days after washout of test reagent. The number of neurons, the number of muscle cells and the total number of differentiated cells were counted after 18-24 hours of culture. Comparison of the number of neurons that differentiated from control and treated embryos showed that inhibition of dopamine β-hydroxylase, the enzyme catalysing the conversion of dopamine to noradrenaline, during the neural plate stages reduced substantially subsequent neuronal differentiation. The differentiation of myocytes and the total number of differentiated cells were not affected. Exogenous noradrenaline (10^{-6} M) or dopamine (10^{-6} M) could increase the number of neurons that differentiated subsequently in culture. Interfering with noradrenaline binding to receptors with receptor antagonists during neurulation showed that α-adrenergic receptor antagonists reduced substantially the subsequent differentiation of neurons. The differentiation of myocytes and the total number of differentiated cells were not affected. The effect of α-adrenergic receptor antagonists was overcome by the simultaneous inclusion of noradrenaline or α-receptor agonists, but not agonists at β-adrenergic receptors. The quantitative reduction in the differentiation of neurons was paralleled by defects in the Central Nervous System of intact tadpoles. The grey matter (containing the cell bodies) was disorganized, although there were no signs of increased cell death, and there was a substantial reduction in the white matter, which contains outgrowing axons. Antagonists at β-adrenergic receptors, dopamine receptors and acetylcholine receptors had no influence on the differentiation of neurons, whether assayed in tissue culture or in intact tadpoles. We suggest that during normal development endogenous noradrenaline binds to α-adrenergic receptors on neural plate cells as part of the mechanism controlling the differentiation of CNS neurons.

Key words: neural, dopamine, noradrenaline, neurulation, differentiation, neuron, *Xenopus*

INTRODUCTION

The events that lie between induction of the nervous system and the appearance of the first differentiated neurons and glia are largely unknown. Induced ectoderm thickens to form the neural plate, which gives rise to the Central Nervous System, outlined by the neural folds. The neural crest, precursor of the peripheral nervous system and other cell types such as melanophores, lies at the top of the neural folds. The neural plate folds in, rolls up and fuses in the midline to form the neural tube, overlaid by the neural crest. In *Xenopus*, separation of neural plate cells into neuronal and glial lineages occurs late, since at neural fold stages a single neural plate cell can generate both neurons and glia (Harten-

stein, 1989). The first differentiated neurons and radial glia appear in the neural tube 3 to 4 hours after closure (N. J. Messenger and Warner, 1989).

The acquisition of an overall neural fate by the ectoderm is reflected by molecular markers such as N-CAM (Kintner and Melton, 1987) and *XlHbox*6 (Sharpe and Gurdon, 1990), although the functional role of such molecules is not known. Physiological studies have revealed alterations in the membrane properties of neural plate cells that are important for the differentiation of neurons. In the amphibian embryo, the membrane potential of neural plate cells increases during the mid-neural fold stages (Warner, 1973), reflecting the activation of additional sodium pumps in neural plate cells (Blackshaw and Warner, 1976). These sodium pumps are inserted into the cell membranes early in neurulation, before they are activated in the normal course of development. Prior to natural activation, they can be switched on by raising extracellular potassium (Blackshaw and Warner, 1976), but the endogenous activating signal has yet to be identified. Identification of this signal would be an important step forward since functional activity of these sodium pumps seems to be essential for the subsequent differentiation of Central Nervous System neurons from the neural plate (E. A. Messenger and Warner, 1979; Breckenridge and Warner, 1982).

The sodium pump is activated by growth factors and neurotransmitters, particularly monoamines (Phillis and Wu, 1981). Catecholamines have been found in the notochord of chick embryos (Lawrence and Burden, 1973). We show here that dopamine, noradrenaline and L-dopa are present in early *Xenopus* embryos. Interfering with the biosynthesis of these monoamines has major consequences for the differentiation of neurons. This makes it plausible that monoamines might activate the sodium pumps in neural plate cells that are important for neuronal differentiation.

However before deciding whether this hypothesis is correct, it is necessary to define the way in which monoamines interact with neural plate cells. The second part of the paper reports such experiments.

MATERIALS AND METHODS

Adult *Xenopus* were induced to mate and lay eggs by injection of chorionic gonadotrophins. Embryos were reared to the required stage (Nieuwkoop and Faber, 1967) at 16-22°C.

HPLC analysis of monoamines

Embryos were stripped of jelly in 2% cysteine in Holtfreter's solution (NaCl: 60 mM; KCl: 1 mM; $CaCl_2$: 0.5 mM; Trishydroxymethylaminomethane: 1 mM) at pH 8 and rinsed in 0.1 M sodium phosphate at pH 7.5. 20 to 40 embryos, with minimum buffer, were transferred to sterile tubes (Nunc), and prepared for analysis or frozen in liquid nitrogen for 10 minutes and stored at −70°C. Each batch gave more than one sample. Samples were split for duplicate or triplicate determinations. On occasion the number of embryos was increased to 60, 80 or, exceptionally, 120. Any further increase introduced interfering contaminants. Monoamine oxidase inhibitors were included during sample preparation.

Embryos were homogenized in 300 µl of 100 mM perchloric acid (PCA) containing 38 ng/ml sodium metabisulphite and 25 ng/ml dihydroxybenzylamine (DHBA) as an internal standard. The homogenate was transferred to a sterile 1.5 ml Eppendorf tube on ice and spun at 3000 ***g*** for 17 minutes at 4°C. The lipid layer was removed and the remaining clear supernatant transferred to a sterile Nunc tube, mixed with 1.0 ml 0.5 M Tris buffer (pH 8.6) and 10 mg acid washed alumina, and shaken at 4°C for 15 minutes. The alumina was washed three times in 1.0 ml 0.1 M Tris buffer (pH 8.6) containing 95 ng/ml sodium metabisulphite and 0.1 mM EDTA and shaken for 1 minute at room temperature in 100 mM perchloric acid with 38 ng/ml sodium metabisulphite (PCA/metabisulphite). The alumina was allowed to settle and an aliquot of alumina-free solution injected directly into the HPLC apparatus. Standards were prepared in the PCA/metabisulphite solution.

Standards (L-dopa, dopamine, noradrenaline and DHBA) of known concentration established the position at which each emerged from the HPLC column. This was followed by a test sample. The identities of the monoamines and breakdown products were confirmed by 'spiking' an experimental aliquot. A small number of samples was run through a system set up for the detection of noradrenaline. We are grateful to Dr C. Stansfield for allowing us to use this equipment.

The HPLC equipment consisted of a Waters 6000-A pump and U-6K injection valve with a µBondpack C18 reversed-phase column (300×3.9 mm ID, 10 µm particle size). The amperometric detection system was made up of glassy carbon working and reference electrodes, a Ag/AgCl reference electrode and an LC-3 electrochemical controller. The working potential was +720 mV.

Recovery was calculated using the internal standard (DHBA) and lay between 20 and 60%. Monoamine concentrations were determined from the height of the peak on the chart compared to standards and corrected for loss during preparation to give the monoamine concentration in the embryos. This was converted to µmole/l embryo, taking embryo volume as 1.2 µl at all stages. Embryo volume can vary (range 1.02-1.5 µl at stage 7; see Slack et al., 1973). However, since each sample contained 20-40 embryos and variation between batches could be considerable, greater accuracy did not seem warranted.

Aldehyde-induced fluorescence of monoamines

Embryos were stripped of jelly with forceps, rinsed for 30 minutes in 0.1 M sodium phosphate, pH 7.6 and prepared using the Faglu technique (Furness et al., 1977), which generates water-stable fluorescent products. Embryos were fixed in 0.5% glutaraldehyde/4% paraformaldehyde in 0.1 M sodium phosphate buffer at pH 7.6 for 3 to 4 hours at room temperature. Penetration was improved by a small hole in the wall of the embryo. Embryos were given three 10 minute washes in 0.1 M sodium phosphate buffer (pH 7.6), and then incubated for 30 minutes in 2.5% sucrose/0.5% sodium azide in phosphate buffer, to prevent cracking. Embryos were soaked in OCT compound (Miles Diagnostics) for at least 30 minutes, orientated in a hollow of frozen OCT and frozen for 10 seconds in isopentane surrounded by liquid nitrogen, attached to a metal chuck with frozen OCT and transferred to a cryostat. After 30 minutes, frozen sections (5-10 µm) were taken, mounted onto gelatinized slides, transferred to an airtight desiccator over silica gel and stored overnight. Sections were mounted in liquid paraffin, viewed on a Zeiss microscope equipped with incident fluorescence and photographed with Kodak Ektachrome 400. Fluorescence was excited at 410 nm and viewed at 475-490 nm, when L-dopa, dopamine and noradrenaline fluoresce with a blue colour. Differentiation between them is difficult since all three emit at a similar wavelength, but they can be distinguished from 5-hydroxytryptamine, which fluoresces yellow, and autofluorescence generated by the yolk platelets, which gave a range of yellow/green colours. This can confuse observation of 5-HT, but is unlikely to complicate observations of the other monoamines. No attempt was made to assign particular colours to individual monoamines; colour reproduction on film was dependent on the fluorescence intensity of the specimen. The effec-

tiveness of the reaction was checked by exposing the sections to a solution of 0.1% sodium borohydride in 90% isopropanol for 2 minutes, followed by 30 seconds in 100% ethanol and three rinses in 85% ethanol. After drying at 50°C for 20 minutes, slides were mounted in liquid paraffin. This removed all blue fluorescence.

Immunocytochemical localization

All solutions contained 10 mg/ml sodium metabisulphite at pH 7.5. Embryos were fixed for 2-3 hours in 1-5% glutaraldehyde in 0.1 M sodium cacodylate and washed for 30 minutes in 0.05 M Tris-HCl buffer at room temperature. They were incubated for 30 minutes in 2.5% sucrose/0.1% sodium azide in phosphate buffer, before preparing frozen sections as above. 5 μm sections mounted on gelatinized slides were stored in an air-tight box at room temperature until staining with the schedule: (i) 5 minutes in buffer with sodium borohydride (1 mg/ml); (ii) 10 minutes in buffer; (iii) 30 minutes in 2% foetal calf serum (Sera-labs) followed by 3× 10 minute washes in buffer; (iv) overnight in rabbit polyclonal anti-dopamine antibody (Affiniti Research products), diluted 1:200 in buffer with 0.5% Triton X-100, 1% foetal calf serum; (v) 3× 10 minute washes in buffer; (vi) 3 hours in biotinylated anti-rabbit antibody (Vector) followed by 3× 10 minute washes in buffer; (vii) 2-3 hours in FITC-Streptavidin (Vector) diluted 1:400-1:800 in buffer with 10% foetal calf serum, and a final 10 minute wash in buffer. Sections were mounted in Citifluor (Citifluor Ltd) to reduce fading. For controls, either the primary antibody was omitted or 100 μg/ml dopamine included. Sections were photographed with T-max 400 film (Kodak). Only the Affiniti Research products antibody to glutaraldehyde-linked dopamine was effective. Patterns consistent with leakage of dopamine from the embryo (e.g. staining only part way through the outer ectoderm layer) were encountered. The results were only considered when stain was evenly distributed through whole cells. Reliability was improved by including formaldehyde in the fixative and increasing the sodium metabisulphite concentration. All observations are based on examination of at least 3, and more usually 6, embryos of the appropriate stage.

Quantitative analysis of neuronal differentiation

A full description is given in E. A. Messenger and Warner (1979). Jelly was removed with forceps from 20 to 40 embryos at stage 13. Embryos were soaked in 3 changes of sterile frog Ringer (NaCl, 120 mM; KCl, 2.5 mM; $CaCl_2$, 2 mM; Tris, 5 mM; pH 7.4) for a total of 30-40 minutes; the final wash contained 100 i.u./ml penicillin/streptomycin (Gibco Ltd). 12 embryos were transferred to each test solution (appropriate drug in Ringer), the remainder to Ringer alone and vitelline membranes removed with forceps. A hole was pierced in the belly to allow entry of reagents. Embryos were treated until the neural folds fused (stage 20) and the test solution washed away. When treatment ended before stage 20, the embryos were returned to Ringer solution and the hole in the belly re-opened to wash away the drug. The embryos were left until they reached stage 20 and prepared for culture. All subsequent steps were identical for both control and treated embryos.

Neural tube, notochord and somites were transferred to dissociating solution (1 mM EGTA in Ca^{2+}- and Mg^{2+}-free Ringer) for 3-5 minutes, reduced to single cells by repeated aspiration and plated (culture Ringer: NaCl, 100 mM; KCl, 2.5 mM; $CaCl_2$, 2 mM; $MgCl_2$, 2 mM; $NaHCO_3$ 5 mM; foetal bovine serum, 10%; penicillin/streptomycin, 100 i.u./ml). 3 embryos gave one culture; cultures were prepared in triplicate. After 18-24 hours at 22°C, most cells had attached and formed a monolayer of differentiated cells. Unattached cells were washed away and the numbers of neurons (phase-bright cell bodies, branching neurites, often with varicosities, and growth cones; cells with this morphology stain with neurofilament protein antibodies and generate action potentials), myocytes and total differentiated cells were counted in at least 21 microscope fields, each containing between 75 and 250 cells, drawn from the 3 culture dishes. Each culture contained approximately 10,000 differentiated cells and about 1000 cells were counted per culture. The results were expressed in two ways: (i) neurons and myocytes were calculated as a percentage of the total number of cells in each field and frequency distributions calculated for control and test in individual experiments; (ii) the absolute numbers of cells in each class (neurons, myocytes and total) were summed over the experiments. The median was determined for each frequency distribution and distributions compared using the Mann-Whitney U-test. $P<0.05$ reflected a significant difference. For individual experiments, the ratio of the medians (median treated population/median control population: T/C) allowed convenient comparison and collation of results from a number of experiments. Data summed over experiments allowed comparison between relatively large numbers (controls for 4 experiments typically gave 600-700 neurons and 4000-5000 myocytes out of about 13,000 cells counted). When summed comparisons were made, a P value of <0.05 reflected differences as small as 10%. In individual experiments $P<0.05$ corresponded to a T/C median ratio of <0.8 (>1.2). When assessing whether neuronal, muscle or total cell numbers had been altered by treatment, the limit was set at 20% (median ratio: 0.8 or 1.2) for both methods.

We compared mixed cultures to determine whether effects were restricted to the neuronal population and to identify cytotoxic treatments. Myocyte differentiation provided a control for another cell type while comparison of total cell numbers revealed cytotoxic effects. Treatments that produced a significant drop in total cell numbers were ignored. There was some variability between cultures prepared from different batches of embryos and control neuron frequency distributions could have a median greater than 10% (relative to the total number of cells in each field), although in most cases it lay between 3.5 and 5% (roughly equivalent to cultures containing about 1700 neurons of which 170 were counted for assay). A similar range was encountered previously (E. A. Messenger and Warner, 1979; Breckenridge and Warner, 1982). When total cell number was low, or when the median control neuron percentage was less than 2% (regardless of absolute numbers of neurons), the experiment was discarded. To avoid observer bias, cultures were frequently counted blind or counted by two separate observers.

Neural differentiation in intact embryos

Embryos were transferred to $^1/_{10}$ Ringer solution after re-opening the belly wall to wash out test solution and left to develop to stage 37-38. Tadpoles were killed by immersion in tricaine (0.1% solution: Sigma), fixed in 4% paraformaldehyde, dehydrated and embedded in methacrylate. 7 μm serial sections were stained with haematoxylin and eosin.

Chemicals

Analar grade (BDH) salts were used. Drugs kindly donated were: benserazide (Roche Products Ltd), carbidopa (Merck, Sharp and Dohme), nomifensine (Hoechst UK Ltd), phenoxybenzamine (Smith, Kline and French Laboratories Ltd), phentolamine (Ciba Laboratories Ltd), prazosin (Pfizer Central Research), HEAT (Dr D. Sugden, Kings College London) and PI-OH (Dr S. Kobayashi, University of Tokushima). Rauwolscine was obtained from Carl Roth. All other chemicals came from Sigma.

RESULTS

The distribution of monoamines and their influence on differentiation

The time course of appearance of monoamines

HPLC analysis was carried out on fertilized eggs, 64-cell

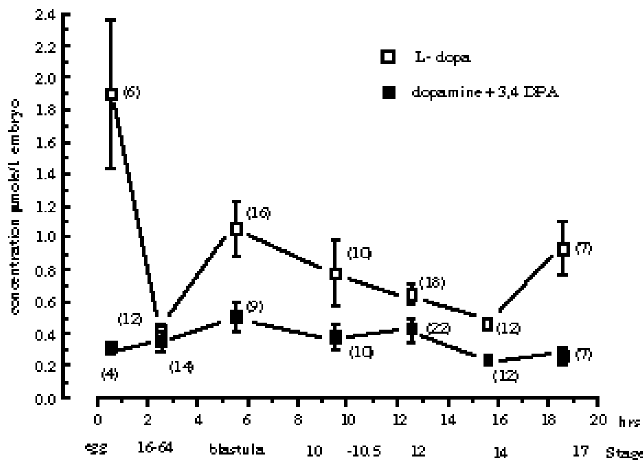

Fig. 1. The concentrations of dopa and dopamine in the early *Xenopus* embryo. Ordinate: monoamine concentration (μmole/l embryo). Abscissa: time after fertilization (hours). The equivalent Nieuwkoop and Faber (1967) stages are given below. Open symbols: L-dopa. Closed symbols: (dopamine + 3,4-DPA). For each point, the error bars give ±1 standard error of the mean. The figures in parenthesis give the number of batches analysed at each stage.

stage embryos, blastulae (NF stages 8 and 9), gastrulae (NF 10-10.5) and during neurulation (NF 12, 14 and 17).

L-dopa, dopamine and its breakdown product, 3,4-dihydroxyphenylacetic acid (3,4-DPA), were detected at all stages. Only 3,4-DPA was identified out of a range of dopamine and noradrenaline breakdown products and precursors. The absence of 3-methoxytyramine and homovanillic acid, generated by catechol-O-methyl transferase, implies that at these stages only monoamine oxidases are available to break down monoamines. 3,4-DPA was rarely absent, suggesting that homogenization released a high concentration of monoamine oxidases, presumably from intracellular stores, and that breakdown of dopamine was not prevented completely.

Fig. 1 plots the average concentrations of L-dopa. Variation was prominent in fertilized eggs and arose from a bimodal distribution, with some samples grouped at above 2×10^{-6} M L-dopa, while others lay around 0.5×10^{-6} M. A bimodal distribution was present at other stages and is reflected in a large standard error. At the 64 cell stage, all batches had L-dopa concentrations towards the lower end of this range, as did those at stages 12 and 14. Some variation could stem from batches with particularly large, or particularly small, diameter eggs, although to encompass the full range, embryo volume would have to differ much more than noted by Slack et al. (1973). It seems more probable that variation reflects inherent differences in the amount of L-dopa synthesized in the oocyte and persisting in the fertilized egg. On this basis, the difference in L-dopa concentration between fertilized eggs and cleavage stage embryos has no significance. From the cleavage stages onwards there was no systematic alteration in L-dopa concentration up to stage 17.

Fig. 1 plots also the concentrations of dopamine, as the sum of dopamine and 3,4-DPA, on the assumption that 3,4-DPA reflects inadequate prevention of dopamine breakdown. Some samples contained only dopamine, while others contained only 3,4-DPA, and dopamine and 3,4-DPA levels were inversely correlated, supporting this assumption. There was little variation in (dopamine + 3,4-DPA) concentration with developmental stage. All samples indicated a concentration of about 2×10^{-7} M dopamine.

Noradrenaline is synthesized from dopamine through dopamine -hydroxylase. Prior to stage 12, noradrenaline was not detected. To improve detection at stages 12 and 14, we increased the number of embryos per sample and used a system set up specifically for noradrenaline. This enhanced reproducibility and noradrenaline was always detected at both stages. However the concentrations were low and the signal-to-noise ratio poor, precluding accurate determination of noradrenaline concentration. Increasing the number of embryos did not improve the situation because interfering contaminants increased with embryo number. We conclude that noradrenaline is present in neurulating *Xenopus* embryos, but at very low concentration.

None of the samples contained 5-HT. The first 5-HT containing neurons appear at stage 26 (N. J. Messenger and Warner, 1989) and 5-HT was first detected by HPLC at stages 27-28.

The location of monoamines within the embryo

Ideally the cellular location of the monoamines detected with HPLC would be revealed with monoamine-specific antibodies. Of the antibodies available, only one dopamine antibody stained undifferentiated ectoderm and mesoderm cells (see below). However, the Faglu technique recognizes noradrenaline and its precursors L-dopa and dopamine. It is relatively easy to distinguish between 5-HT and noradrenaline or its precursors, but difficult to separate dopa, dopamine and noradrenaline. It was almost impossible to ensure uniform colour reproduction on film from specimen to specimen because variations in levels of fluorescence altered the absolute colour recorded.

Fig. 2A shows a frozen section through a blastula stage embryo. Blue fluorescence, indicating the presence of L-dopa, dopamine or noradrenaline, was apparent in animal pole cells but was less pronounced, or even absent, within the vegetal pole, where individual yolk platelets fluoresced with a variety of yellow/green tones. This arises almost certainly from autofluorescence. It is unlikely to reflect 5-HT, which was not detected at these stages with HPLC. Treatment with sodium borohydride eliminated the blue fluorescence. A similar distribution of monoamine fluorescence was apparent in all blastulae examined.

Fig. 2B shows a section through the dorsal region of a gastrulating embryo at stage 10.5. The inner ectoderm and mesoderm layers showed predominantly blue fluorescence, which was less prominent in the outer ectoderm. The inner, yolky endodermal cells showed pale yellow/green autofluorescence in the yolk platelets. Fig. 2C shows the dorsal region of an embryo at stage 14; again the ectomesodermal location of blue monoamine fluoresence is apparent. After exposure to sodium borohydride all regions show very low levels of fluorescence with very dark, green colour (Fig. 2D).

Immunocytochemical localization of dopamine

Fig. 3 shows frozen sections through dorsal, lateral and ventral regions of embryos at stages 12, 14 and 17 stained

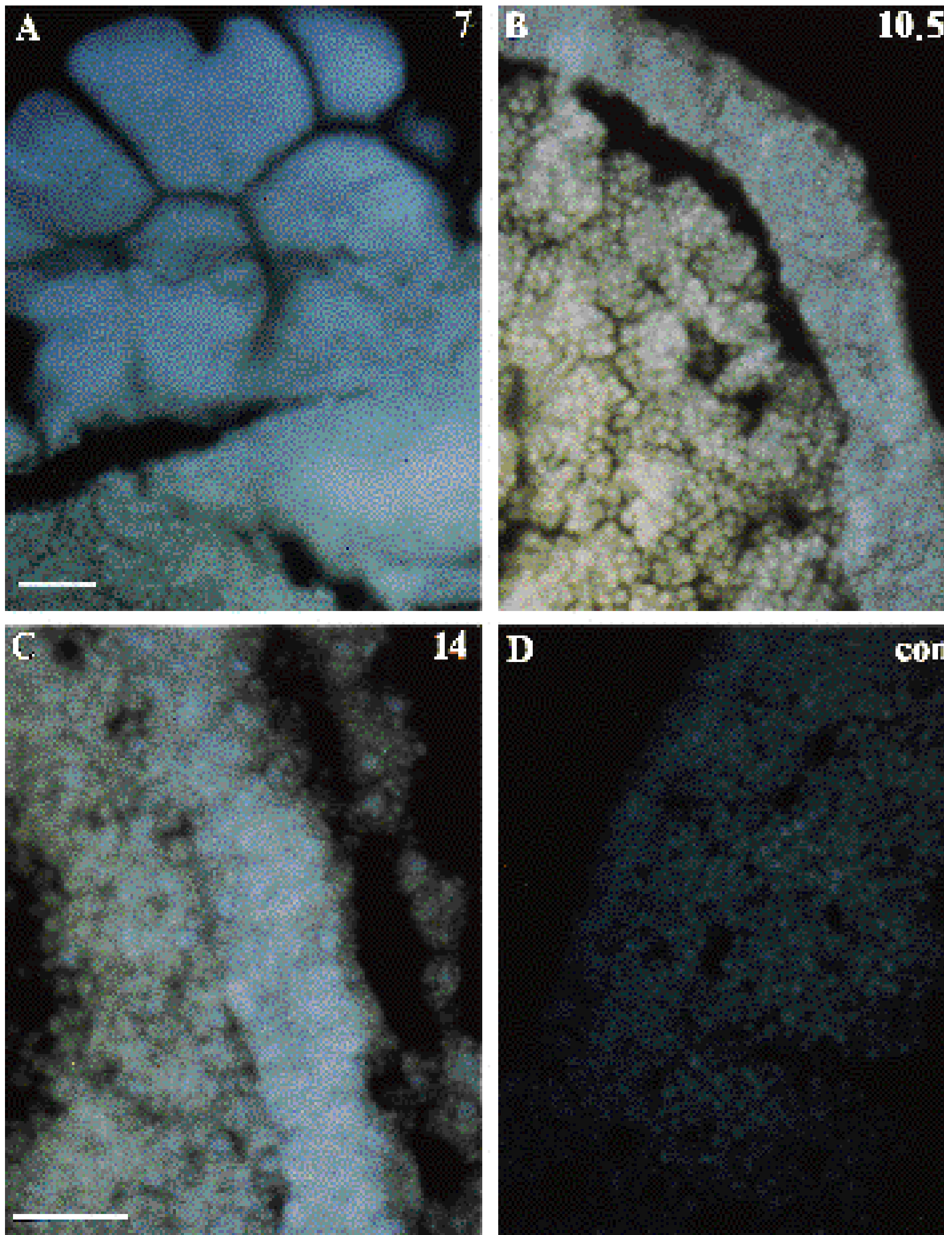

Fig. 2. Monoamine fluorescence induced by the Faglu technique. Each panel shows part of a frozen section through an individual embryo. (A) Stage 7 (blastula). Note bright blue fluorescence indicating the presence of dopa, dopamine or noradrenaline within cells of the animal pole. The bottom of the photograph shows part of the endoderm, where the large yolk platelets fluoresce with green autofluorescence. (B) Embryo at stage 10.5. Monoamine fluorescence is prominent in the deep ectoderm and mesodermal layers of the embryo. The outer ectoderm shows patchy blue fluorescence, while the endoderm shows predominantly autofluorescence. (C) Embryo at the early neural fold stage (NF stage 14). Blue fluorescence is again restricted to the deep ectoderm and mesoderm layers. The clumps of yolk platelets (the surrounding cell membranes are not visible) show autofluorescence. (D) Section from embryo at stage 14 that had been treated with borohydride to abolish monoamine fluorescence. Intrinsic fluorescence was so low that colour has been rendered dark green. Bar, 100 μm.

with an antibody to dopamine, together with controls in which dopamine competed out antibody staining. Dopamine-specific staining was predominantly cytoplasmic, the yolk platelets and other organelles appearing dark against the cytoplasm. In dopamine-negative regions, the yolk platelets stand out against the unstained cytoplasm. The controls also showed yolk platelets to be relatively bright (Fig. 3D,H,M). Dopamine was excluded from the endoderm, confirming the Faglu staining. This is particular clear in Fig. 3B where a section at stage 12 shows completely negative yolk plug cells, which are endodermal, with dopamine staining in the flanking dorsal ectoderm.

Dopamine staining showed a dorsoventral gradient. At stage 12, dopamine was present predominantly in the dorsal outer ectoderm (A) while ventral ectoderm was negative (C). At stage 14, when the neural folds are lifting, dopamine staining increased and in dorsal regions was present in both ectoderm and mesoderm (Fig. 3E). Laterally, dopamine was present only in the ectoderm (F), while ventrally (G) stain was patchy and weak. At stage 17, the neural plate, notochord and somitic mesoderm contained dopamine (I). Staining stopped abruptly at the edge of the somitic mesoderm (J). Laterally dopamine was restricted to the ectoderm (K) and staining of the outer ectoderm layer only was apparent in ventral regions (L).

Dopamine-specific staining was more restricted than the fluorescence induced by Faglu, which cannot distinguish between the monoamines. Since HPLC analysis showed noradrenaline concentrations to be very low and put the concentration of L-dopa consistently higher than dopamine, it is probable that the bulk of the monoamine fluorescence seen with Faglu stems from L-dopa.

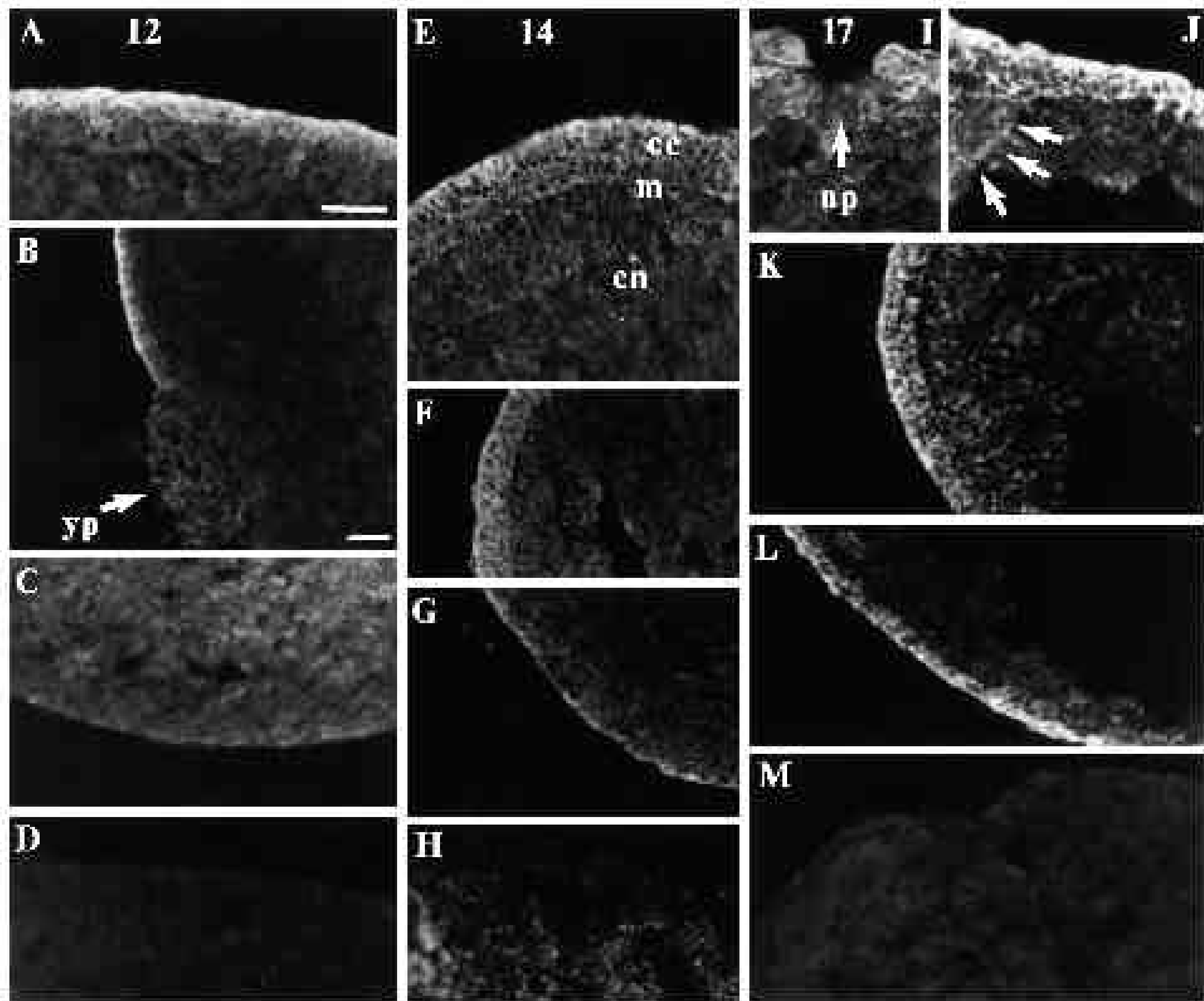

Fig. 3. The immunocytochemical localization of dopamine during the neurula stages: 12 (A-D); 14 (E-H) and 17 (I, J, K-M). The upper panels (A,E,I,J) show dorsal regions of the embryo. Note that at stage 12 (A) dopamine is present in the ectoderm only. At stage 14 (E) dopamine is present in the neural ectoderm and the underlying dorsal mesoderm, but not in the endoderm. At stage 17 (I,J) the neural plate and dorsal mesoderm are brightly stained. Mesodermal localization of dopamine terminates abruptly at the lateral edge of the somitic mesoderm (arrows). The middle panels (B,F,K) show lateral regions. Note that at stage 12 (B) dopamine is present only in the dorsal ectoderm flanking the yolk plug. At stages 14 (F) and 17 (K) both superficial and deep ectoderm cells contain dopamine. In ventral regions (C,G,L), dopamine is absent at stage 12 (C), patchy at stage 14 (G) and in the outer ectoderm only at stage 17 (L). The bottom panels (D,H,M) show sections from embryos where dopamine-specific antibody staining has been competed out by 10^{-6}M dopamine. Note either complete absence of fluorescence (D,M) or autofluorescent staining of yolk platelets only (H). Bar, 100 μm.

We were unable to demonstrate staining with antibodies to either noradrenaline or dopamine -hydroxylase. This could reflect the low concentration of noradrenaline indicated by HPLC; we have no independent estimates of the -hydroxylase concentration, but this also could be very low. Alternatively the available antibodies may not recognize epitopes exposed when noradrenaline and its synthetic enzyme are present in early embryonic cells rather than in neurotransmitter storage vesicles in neurons.

Inhibitors of catecholamine biosynthesis influence neuronal differentiation

If catecholamines function as early developmental signals, preventing their biosynthesis should perturb development. L-dopa is converted to dopamine by L-dopa decarboxylase; dopamine -hydroxylase catalyzes the conversion of dopamine to noradrenaline. The number of neurons that differentiated in cultures prepared from embryos treated with monoamine synthesis inhibitors during neurulation was compared with the number that differentiated from sham-treated siblings. The drugs were present only during neurulation (Nieuwkoop and Faber stages 14 to 20; approx. 4 hours).

Fig. 4 compares frequency distributions for the number of neurons differentiating in cultures prepared when the neural tube had just closed (see Materials and Methods) from control embryos (A) and embryos that had been treated during neurulation with the dopamine -hydroxylase

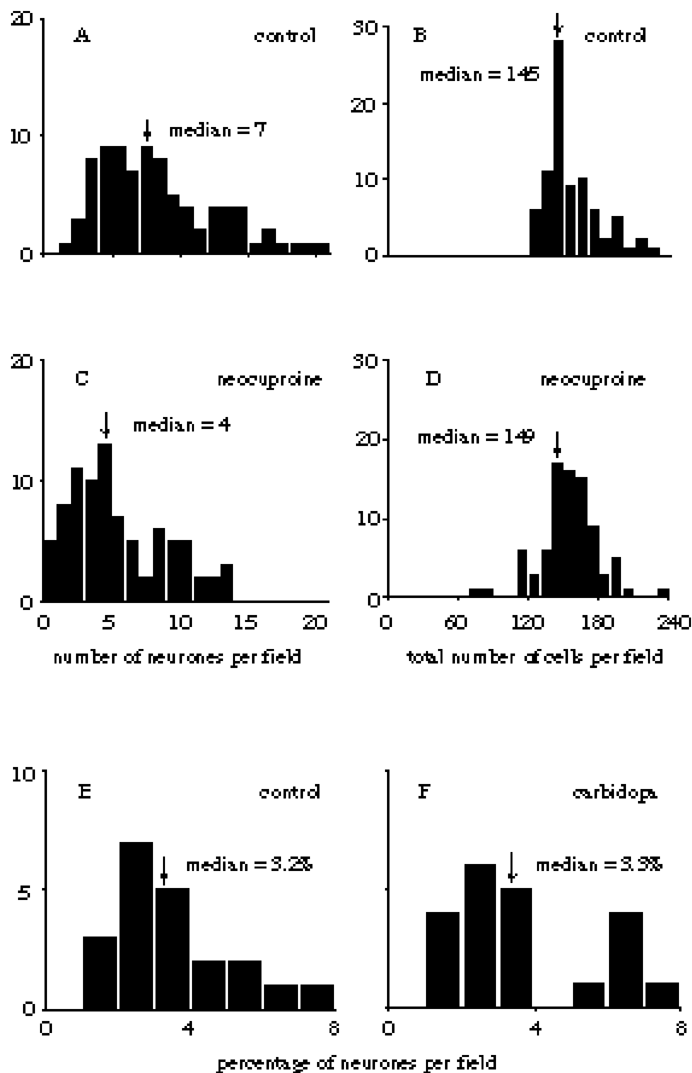

Fig. 4. (A-D) The consequences for neuronal differentiation of inhibiting dopamine -hydroxylase in neurulating embryos. Sum of results from 4 different experiments. Ordinates: number of fields (*n*). Abscissae: number of cells in each microscope field. (A) Control frequency distribution for absolute numbers of neurons differentiating in cultures prepared when the neural tube is closed. (C) Frequency distribution for neurons differentiating from embryos treated with 5×10^{-6} M neocuproine during neurulation. A versus C P<0.0001. (B,D) Frequency distributions for total cell numbers in cultures from control and treated embryos. B versus D P>0.2. Note that neuron numbers are significantly reduced whereas total cell numbers are unaffected. (E,F) Inhibition of dopa decarboxylase during neurulation does not affect neuronal differentiation. Ordinates number of fields (*n*). Abscissae: neurons in each field as a percentage of the total number of cells. E, control; F, carbidopa (10^{-6} M). Arrows indicate the median of each distribution.

inhibitor neocuproine (5×10^{-6} M; Green, 1964; C) summed over 4 experiments. In the control, only 15% of the fields contained fewer than 4 neurons and 30% had more than 10 neurons. The number of neurons differentiating after treatment with neocuproine was reduced significantly. 40% of the fields now contained fewer than 4 neurons and only 14% contained more than 10. The maximum number of neurons per field fell from 20 to 13. The frequency distribution was shifted significantly to lower levels of neuronal differentiation (Mann-Whitney U-test A versus B P<0.0001). The total number of cells was not affected (B,D) indicating that inhibiting dopamine -hydroxylase had no non-specific cytotoxic effects. The ratio of the medians (test/control) was 0.57, indicating a substantial reduction in the neuronal population. In the 4 experiments that provided the summed data, the ratio of the medians treated/control was 0.55 (±0.1 s.e.m.). Four experiments with the inhibitor diethyldithiocarbamate (2×10^{-6} M) gave a median ratio of 0.57 for summed data and 0.65 (±0.06 s.e.m.) for individual experiments. Both methods of analysis show a substantial and significant reduction in neuronal differentiation. There was a small difference in myocytes when the data were summed (approx. 12%). This was not apparent in individual experiments, where the test/control median ratio was never less than 0.85. In contrast to the neuronal population, cells destined to be myocytes were insensitive. The reduction in neuronal differentiation observed after dopamine -hydroxylase inhibition was prevented when noradrenaline was included with the -hydroxylase inhibitor.

To check that the reduction in neuronal differentiation was not the result of carry-over into the cultures, either 5×10^{-6} M neocuproine or 2×10^{-6} M diethyldithiocarbamate was added at the time of preparation of cultures from previously untreated embryos. Neither inhibitor was effective (neocuproine; T/C=0.83±0.09 s.e.m., *n*=4: diethyldithiocarbamate; T/C=0.93±0.1 s.e.m., *n*=4).

Sibling embryos, allowed to develop for two days after washout of the -hydroxylase inhibitors, were examined for neural defects. These were identical to those observed after other treatments that reduce neuronal differentiation and are described with them.

Inhibition of L-dopa decarboxylase (Carlsson, 1964; Modigh, 1973) had no influence on neuronal differentiation, even when treatment began at the end of gastrulation. Fig. 4E,F shows one experiment with the L-dopa decarboxylase inhibitor carbidopa where control and test frequncy distributions are identical, drawn from 22 experiments with three L-dopa decarboxylase inhibitors (benserazide, carbidopa and 3-hydroxybenzylhydrazine) both alone or in combination, at concentrations ranging from 5×10^{-7} M to 10^{-4} M. Even when data were summed to see whether a small effect might then be revealed, no effect could be demonstrated (P>0.5).

The ability of exogenous dopamine and noradrenaline to increase neuronal differentiation was tested also. Five experiments with 10^{-6} M dopamine gave a mean test/control ratio of 1.23 (±0.2 s.e.m.). One showed a large and statistically significant increase (T/C ratio 1.88; P<0.05), suggesting that exogenous dopamine could increase the number of neurons that differentiated, although it did not always do so. There was no difference between the control and test muscle populations (P>0.33; Mann-Whitney test) or the total populations (P>1) (both summed data and individual experiments).

For noradrenaline (10^{-6} M) the results were similar. In five experiments, the mean test/control median ratio was 1.25 (±0.19 s.e.m.); there was no difference between

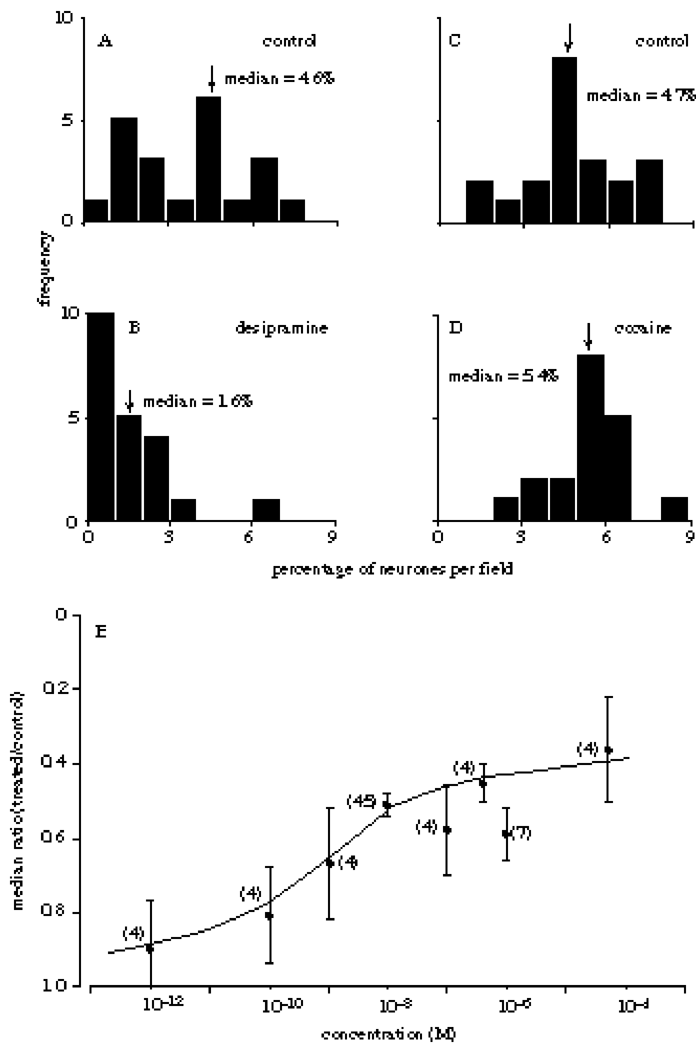

Fig. 5. The consequences for neuronal differentiation of treatment with monoamine uptake inhibitors during neurulation. Two separate experiments. Ordinates: number of fields (*n*). Abscissae: % neurons in each microscope field. (A,C) Frequency distribution for neurons differentiating in cultures prepared at stage 20 from control embryos for the two experiments. (B) Frequency distribution for neurons differentiating from embryos exposed to 10^{-8} M desipramine during neurulation. A versus B P=0.0006. Note the significant reduction in neuronal differentiation. (D) Neurones differentiating from embryos treated with 10^{-6} M cocaine during neurulation. C versus D P>0.2. Note that neuronal differentiation is unaffected. Arrows indicate the median of each distribution. (E) Dose-response relation for the effect of desipramine on differentiating neurons. Ordinate: ratio of the medians test/control (T/C ratio). Abscissa: concentration of desipramine present during neurulation. Bars give the standard error of the mean. The number of experiments at each concentration is given in parentheses.

myocyte number or the total number of cells. In one experiment, there was a substantial and highly significant potentiation of neuronal differentiation (T/C: 1.76; P<0.009), with little overlap between the populations. In the control the median of the distribution lay at 8 neurons per field. In the cultures prepared from noradrenaline-treated embryos, only two fields had fewer than 10 neurons, the majority more than 13 neurons, and the median lay at 14.

The mechanism by which monoamines affect neuronal differentiation

To assess the possibility that monoamines activate the sodium pump during the neural plate stages, we examined how monoamines might interact with neural plate cells.

Do monoamine uptake inhibitors influence neuronal differentiation?

Fig. 5 compares neurons differentiating in cultures from control embryos (A) and embryos that had been treated during neurulation with the noradrenaline uptake inhibitor desipramine (10^{-8} M: B). The distributions were highly significantly different (Mann-Whitney U-test, P=0.0006; T/C ratio 0.35). For 45 experiments, the ratio of the medians 10^{-8} M desipramine/control was 0.51±0.03 s.e.m. Myocyte differentiation and total cell number were not affected. Desipramine had no effect when added at the time of preparation to cultures from previously untreated embryos (10^{-6} M; T/C=0.91±0.12 s.e.m., n=5). Desipramine must be present during neurulation if neuronal differentiation is to be affected.

Fig. 5E plots the ratios (treated median/control median) against the concentration of desipramine present during neurulation. The relationship is sigmoid, with the threshold for a significant reduction in subsequent neuronal differentiation at 10^{-10} M; 10^{-8} M desipramine approached the maximally effective dose. Desipramine never abolished completely neuronal differentiation and approximately 30-35% of the population persisted, as found for inhibitors of the sodium pump (E. A. Messenger and Warner, 1979; Breckenridge and Warner, 1982). Even at its most effective, desipramine did not reduce the T/C ratio to below 0.24. The noradrenaline uptake inhibitor imipramine also reduced significantly neuronal differentiation (10^{-6} M: median ratio T/C=0.58±0.1 s.e.m., n=6; minimum T/C ratio 0.26; threshold 10^{-7} M).

However, the noradrenaline uptake inhibitors cocaine (10^{-6} M) and PI-OH (4-hydroxy-2-methyl-4-phenyl-1,2,3,4-tetrahydroisoquinoline, 10^{-6} M; Ishida et al., 1988) were without effect. Cocaine failed to reduce subsequent neuronal differentiation (T/C=1.09±0.06 s.e.m.; n=10). One of these experiments is shown in Fig. 5C,D. The dopamine uptake inhibitor nomifensine (10^{-6} M) also failed to reduce neuronal differentiation (T/C=0.92±0.12, n=6).

Desipramine and imipramine are -adrenergic receptor antagonists (U'Prichard et al., 1978) as well as uptake inhibitors. The other uptake inhibitors have no antagonistic action at -adrenergic receptors and had no influence on neuronal differentiation.

The consequences of blockade at -adrenergic receptors

Table 1 compares the consequences of treating neurulating embryos with antagonists at 6 different receptor classes, presented as the means (±s.e.m.) of individual experiments. Data summed over the experiments (about 1000 neurons in controls) gave closely similar results. Myocyte differentiation and the total numbers of differentiated cells were not

Table 1. The effects of receptor antagonists on neuronal differentiation

Receptor class	Antagonist	Concentration (M)	T/C mean±s.e.m.	*n*
adrenergic				
$_1$	prazosin	10^{-6}	0.62±0.06	7
		10^{-8}	0.60±0.05	9
$_1$	HEAT	10^{-6}	0.60±0.10	4
$_2$	rauwolscine	10^{-6}	0.63±0.06	8
		10^{-8}	0.68±0.06	4
$_2$	yohimbine	10^{-6}	0.70±0.09	5
	phenoxybenzamine	10^{-6}	0.76±0.11	7
	phentolamine	10^{-6}	0.83±0.05	5
-adrenergic	propranolol	10^{-6}	0.90±0.10	4
dopaminergic	haloperidol	10^{-6}	0.88±0.17	4
	sulpiride	10^{-6}	0.98±0.06	4
cholinergic				
nicotinic	curare	10^{-6}	0.89±0.10	6
muscarinic	atropine	10^{-6}	1.11±0.16	4

Embryos were treated at the concentrations shown from stage 14 to stage 20, at which point the drug was washed out and the dorsal tissue dissociated and cultured. Neurone numbers in cultures from control and treated embryos were compared using the Mann-Whitney U-test and the median ratio (treated/control) calculated. Mean T/C ratios ± standard error of the mean for a number of experiments (n) are given. For individual experiments, a T/C ratio of 0.80 or less reflects a significant reduction in neurone numbers. None of the reagents had any effect when added to the medium of cultures prepared from previously untreated embryos.

Note: yohimbine, phenoxybenzamine and phentolamine were less effective than the other α-adrenergic receptor blockers and, in some experiments, neuronal differentiation was not inhibited significantly.

affected. Prazosin, a specific $_1$-adrenergic receptor antagonist, and rauwolscine, an $_2$-adrenergic receptor antagonist, both reduced significantly neuron number. Fig. 6 shows examples of experiments with 10^{-8} M prazosin (A,D) and 10^{-6} M rauwolscine (B,E). Each antagonist produced a significant reduction in neuronal differentiation. Prazosin was effective also at 10^{-9} and 10^{-10} M. Neither specific -adrenergic receptor blocker was as efficient as desipramine, with minimal T/C ratios of 0.42 for prazosin and 0.39 for rauwolscine. Increasing the concentration did not improve their effectiveness. The $_1$-adrenergic receptor antagonist HEAT (2-[-(4-hydroxyphenyl)ethylaminomethyl]tetralone HCl; Heinz and Hofferber, 1981; Göthert et al., 1980) was equally able to reduce neuronal differentiation. The $_2$-adrenergic receptor blocker yohimbine was less effective, although on one occasion the T/C median ratio fell to 0.44. The -adrenergic receptor blockers phenoxybenzamine and phentolamine were less effective than prazosin or rauwolscine (see Table 1). Phenoxybenzamine reduced neuronal differentiation significantly in only 3 out of 7 experiments (one is included in Fig. 8), while phentolamine was effective in only 2 out of 5 tests.

Table 1 shows also the effects of antagonists at -adrenergic receptors, dopamine receptors and acetylcholine receptors. None had any influence on neuronal differentiation. Fig. 6C,F shows similar control and test frequency histograms for neurons differentiating after treatment with the -adrenergic receptor blocker propranolol (10^{-6} M).

Monoamines overcome the consequences of -adrenergic receptor blockade

L-dopa, dopamine and noradrenaline all overcame the reduction in neuronal differentiation brought about by desipramine, prazosin and rauwolscine. The results are summarized in Table 2 and examples of rescue brought about by noradrenaline are shown in Fig. 7. The -adrenergic receptor agonist phenylephrine and the specific, but relatively weak, agonist methoxamine also prevented desipramine from inhibiting neuronal differentiation (Table 2). By contrast, the -adrenergic receptor agonist isoprenaline could not overcome the effects of desipramine (median ratio (desipramine + isoprenaline)/desipramine = 1.0±0.09 s.e.m.; *n*=11).

When the conversion of L-dopa to dopamine was prevented with the dopa decarboxylase inhibitor 3-hydroxybenzylhydrazine, exogenous L-dopa no longer prevented the inhibitory effect of desipramine (Table 3). When dopamine -hydroxylase activity was inhibited with neocuproine, exogenous dopamine could not overcome the effect of desipramine (Table 3). This implies that exogenous L-dopa and dopamine must be converted to noradrenaline. The ability of noradrenaline to compete with desipramine was not affected by the dopamine -hydroxylase inhibitor neocuproine (Table 3). The large standard error in the presence of noradrenaline arises because in one experiment the desipramine effect was rather small and noradrenaline then potentiated neuronal differentiation well above the control. A significant agonist-induced potentiation was not uncommon and frequently occurred when the reduction in neuronal differentiation produced by alpha blockade was relatively small. Both phenylephrine and methoxamine could increase neuronal differentiation above control levels, as found for dopamine and noradrenaline.

The time of sensitivity to -adrenergic receptor blockade

Fig. 8 compares the effect of 10^{-6} M desipramine between stages 14 and 17 with its effects at stages 15 to 18 and 17 to 20 for 4 experiments (approximately 4 hours from 14 to 20). Two experiments from a series of different design with desipramine, prazosin, rauwolscine, yohimbine and phenoxybenzamine are shown also in Fig. 7. All showed that -adrenergic receptor blockade at any time during the neural fold stages reduced significantly subsequent neuronal differentiation. This contrasts with inhibiting the sodium pump where the developmental consequences were confined to a narrow, 2 hour time window covering the mid-neural fold stages, 14 to 17 (E. A. Messenger and Warner, 1979).

Assay in whole embryos

Treated embryos and their sibling controls (at least 6 for each treatment) were left to develop in $^1/_{10}$ Ringer, after washout of drug or Ringer solution, until they reached stage 37-38 and examined histologically. A disturbance of the nervous system was apparent for all treatments where neuronal differentiation, assayed in tissue culture, had been reduced. The similarity of the defects produced by the different agents capable of preventing neurons from differentiating was striking. The in vivo consequences of treatment during neurulation with reagents that interfered with noradrenaline metabolism or binding were indistinguishable from each other and from those observed after

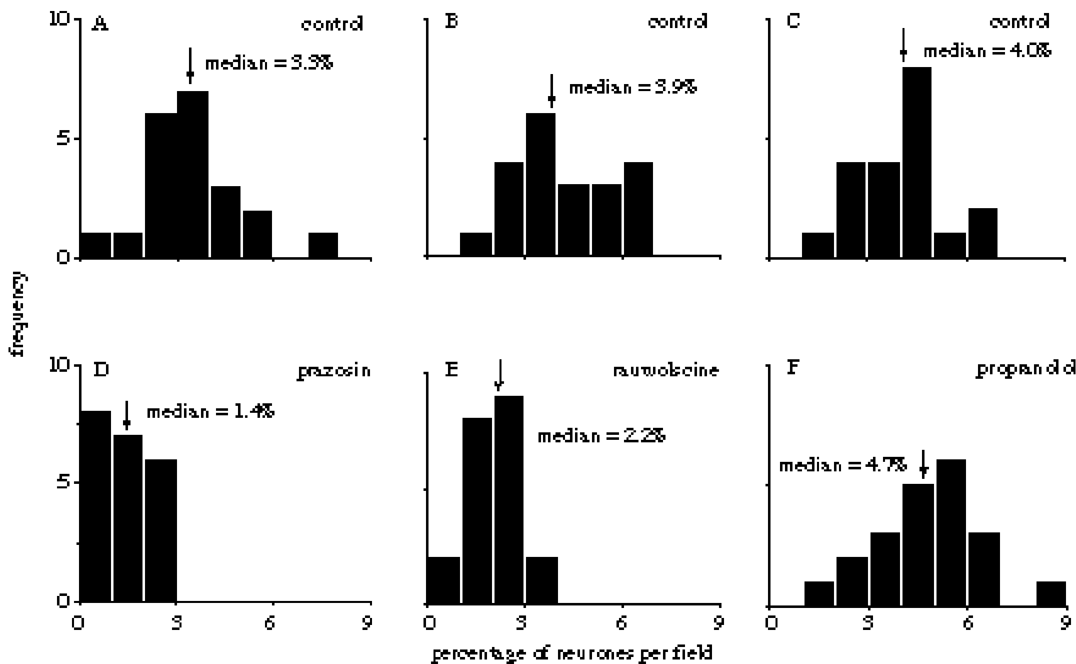

Fig. 6. The consequences for neuronal differentiation of treatment of neurulating embryos with receptor blockers. Three separate experiments. Ordinates: number of fields (n). Abscissae: % neurons in each microscope field. In each case, the arrow gives the median of the distribution. (A,B,C) Control frequency distributions for the three experiments. (D) Frequency distribution for neurons differentiating from embryos exposed to 10^{-8} M prazosin during neurulation. A versus D $P< 0.0001$. (E) Frequency distribution for neurons differentiating from embryos exposed to 10^{-6} M rauwolscine during neurulation. B versus E $P<0.0001$. (F) Frequency distribution for neurons differentiating from embryos treated with 10^{-6} M propranolol during neurulation. C versus F $P>0.2$. Note that only -adrenergic receptor blockers reduce neuronal differentiation. Arrows indicate the medians.

Table 2. Monoamines and α-adrenergic receptor agonists overcome the effects of α-adrenergic receptor blockade on neuronal differentiation

Antagonist	Alone	+ L-dopa 10^{-6}M	+ DA 10^{-6}M	+ NA 10^{-6}M	+ methox-amine 10^{-6}M	+ phenyl-ephrine 10^{-6}M	n
desipramine	0.69±0.11*	1.05±0.07					7
10^{-8} M	0.55±0.07		0.94±0.09				9
	0.71±0.08			1.22±0.26			4
	0.65±0.06				1.01±0.12	1.02±0.01	4
prazosin 10^{-6} M	0.59±0.04			1.07±0.05			8
rauwolscine 10^{-6} M	0.69±0.09			1.16±0.07			4

*All values give the average ratio median treated population/median control (T/C) ±1 standard error of the mean.

blocking the sodium pump (see E. A. Messenger and Warner, 1979; Breckenridge and Warner, 1982). The neural retina and the brain showed substantial cellular disorder and reduced white matter. The volume of grey matter, containing the cell bodies, was not reduced and in some cases was clearly increased. Abnormalities were restricted to CNS derivatives. The relative anterior-posterior positions of brain structures were approximately the same in control and treated tadpoles, suggesting that there was no major disturbance of patterning. The generation and distribution of pigment cells, which are derived from the neural crest rather than the neural plate, was not affected.

Fig. 9 compares the eyecup of a control tadpole (A) with that of a sibling treated during neurulation with the dopamine -hydroxylase inhibitor diethyldithiocarbamate (10^{-6} M: B), a potent inhibitor of neuronal differentiation. In the control (A) the layering of the neural retina is obvious and the adjacent region of the brain shows ordered cells in the grey matter and substantial peripheral white matter. In the diethyldithiocarbamate-treated tadpole (B), both brain and neural retina contain many cell bodies, but there is no order. There is little white matter in peripheral regions of the brain and no sign of developing neural layers in the retina. Fig. 9 compares a control (C) with a sibling that had been exposed to desipramine (10^{-8} M: D). In the control, the organized, emerging layers of the neural retina are easily distinguished. In the embryo exposed to desipramine during neurulation, the neural retina is disorganized and axonal outgrowth from developing neurons appears to be absent. There are no signs of necrosis, suggesting that cell death was not a major contributor. The lens and outer pigment layer, neither of which is derived from the neural plate, were unaffected.

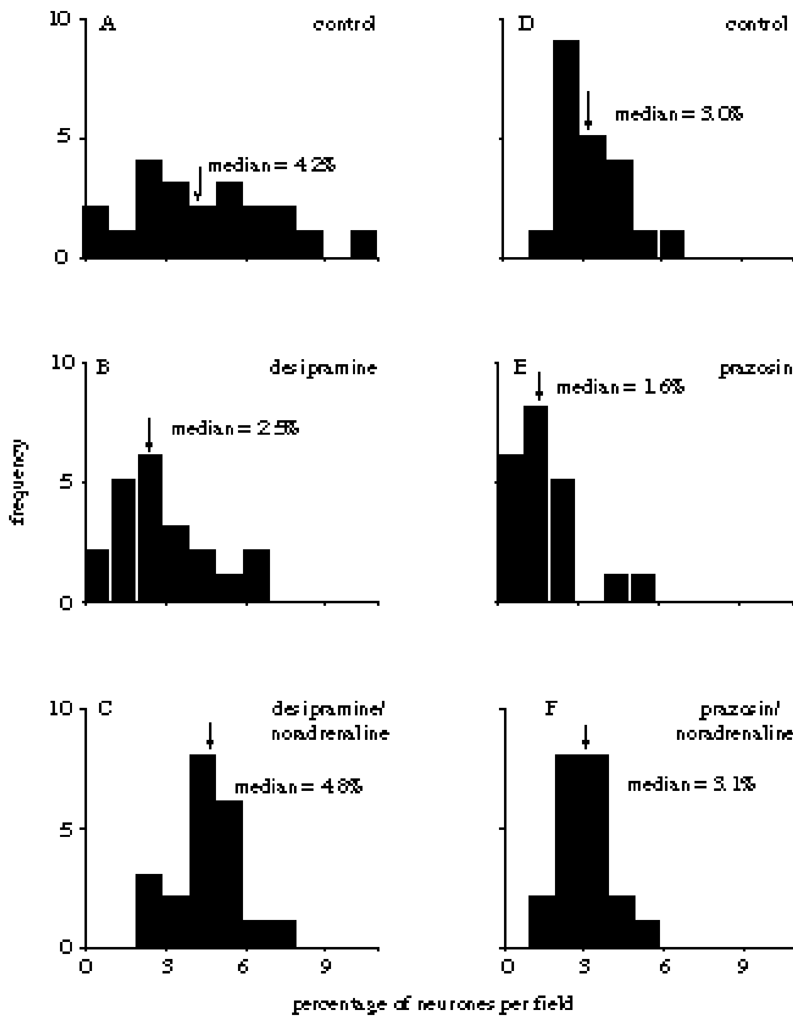

Fig. 7. Noradrenaline can overcome the inhibitory effect of desipramine and prazosin. Two separate experiments. Ordinates: number of fields (*n*). Abscissae: % neurons in each field. The top two panels (A,D) give the control distributions for each experiment. (B) Frequency distribution for neurons differentiating after treatment with 10^{-8} M desipramine. A versus B $P<0.05$. (C) Neurones differentiating after treatment with 10^{-8} M desipramine together with 10^{-6} M noradrenaline. A versus C $P>0.2$. (E) Frequency distribution for neurons differentiating after treatment with 10^{-6} M prazosin. D versus E $P<0.0001$. (F) Neurones differentiating after treatment with 10^{-8} M prazosin and 10^{-6} M noradrenaline. D versus F $P>0.2$. Note that in both cases neuron numbers are restored to control levels by the inclusion of noradrenaline. The medians are indicated with arrows.

Table 3. L-dopa and dopamine cannot compete with α-adrenergic receptor antagonists when noradrenaline biosynthesis is prevented

Desipramine 10^{-8}M	+ L-dopa 10^{-6} M	+ L-dopa + 3-HBH* 10^{-6} M	+ DA 10^{-6} M	+ DA + NC** 5×10^{-6} M	+ NA 10^{-6} M	+ NA + NC 5×10^{-6} M	*n*
0.69±0.11	1.05±0.07	0.56±0.11					7
0.58+/-0.10			1.01±0.10	0.49±0.10			4
0.65+/-0.09					1.33±0.39	1.17±0.17	4

*3-hydroxybenzylhydrazine.
**neocuproine.

The final panels compare a control (E) and a sibling treated with 10^{-6} M propranolol, a -receptor blocker (F) that had no effect on neuronal differentiation. Both control and treated tadpoles are normal and indistinguishable.

DISCUSSION

Monoamines are present in early *Xenopus* embryos

Early *Xenopus* embryos contain L-dopa and dopamine. The concentrations of the two monoamines change little between the fertilized egg and the late neurula stage. Noradrenaline is present at low concentration during the early neurula stages. Monoamines were located predominantly in the ectoderm and mesoderm. An ectodermal/mesodermal localization was found also for dopamine. Although the cellular location of noradrenaline could not be defined, it is likely to be similar to its precursor dopamine. The finding that L-dopa and dopamine, and presumably noradrenaline, are present in dorsal ectoderm and mesoderm, but not in the endoderm, makes plausible a role for these monoamines in the early development of the nervous system. The localization of monoamines was less restricted than in the chick, where endogenous catecholamine fluorescence was found only in the notochord (Lawrence and Burden, 1973). In *Xenopus*

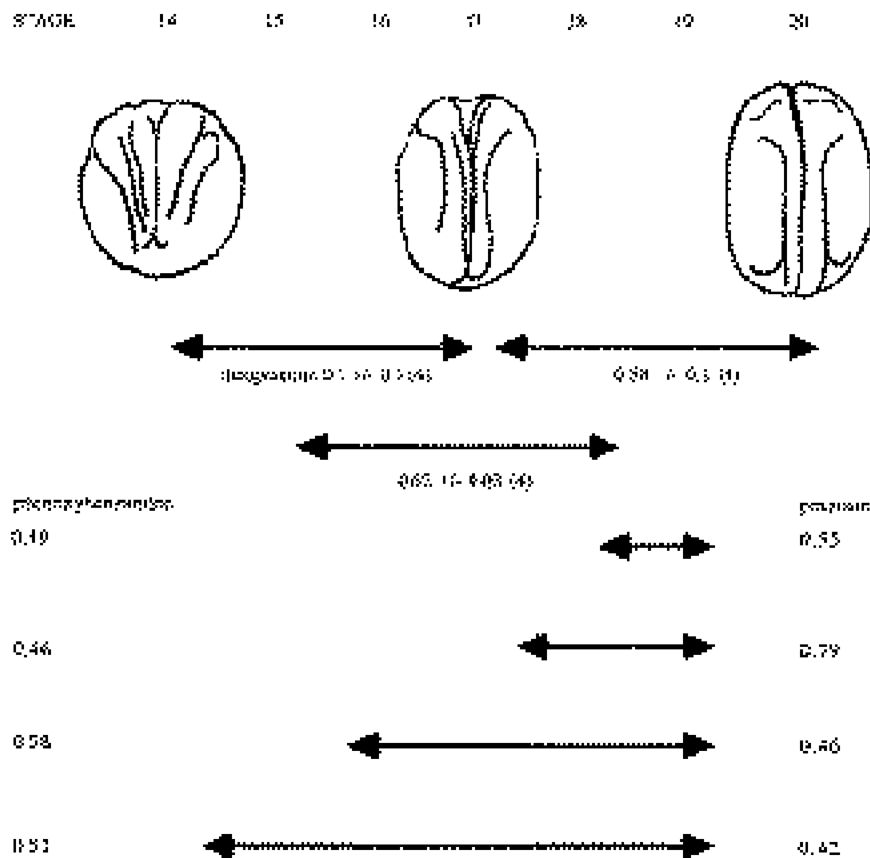

Fig. 8. The time of sensitivity to -adrenergic receptor blockade. The top panel shows embryos at three different stages of neurulation. The horizontal arrows represent the period over which treatment occurred. Note that all three of the -adrenergic blockers shown inhibited neuronal differentiation equally effectively, regardless of the length of time of treatment.

dopa and dopamine are present at relatively high concentration and monoamine oxidases are abundant also, since it proved difficult to prevent breakdown of dopamine. How are these monoamines stored? At the light microscope level, there was no obvious particulate distribution. Dopamine was excluded from the yolk platelets, but uniformly distributed in the cytoplasm. Dense cored vesicles, such as those found in monoamine-containing neurons, are not apparent in the early *Xenopus* embryo. Both published micrographs (e.g. Kalt, 1971) and unpublished material at a range of stages (personal observations by A. E. W.) do not indicate any obvious storage compartments. Nevertheless, there must be efficient separation of monoamines and their oxidases during early development. *Xenopus* embryonic cells contain many membrane-bound organelles: lipid droplets, pigment granules and other clear vesicles, any of which might provide storage sites. The storage capacity of *Xenopus* embryonic cells has previously been recognized (see Slack et al., 1973).

Monoamines are involved in the control of neuronal differentiation

The differentiation of neurons was monitored in two ways. First, the number of neurons that differentiated in culture was compared between embryos treated during neurulation with test agents and sham-treated siblings. Second, the histological organization of the CNS was examined two days after withdrawal of drug. The culture method was developed to assay quantitatively the consequences of inhibiting the sodium pump during neurulation (E. A. Messenger and Warner, 1979; Breckenridge and Warner, 1982). There is some variability between experiments, deriving in part from rapid healing of the belly ectoderm, which can terminate penetration into the intercellular spaces before equilibration with the bathing solution. The limit for detection of significant differences between treatments within a single experiment was set at 20%, which contributes to the variability, but reflects a balance between the number of cells that could be counted within a reasonable time (about 15,000 cells) and comparisons of more than one treatment in each experiment. The assay has the advantage that neuronal differentiation can be assessed quantitatively after treatment of intact, neurulating embryos. Morphological examination of tadpoles derived from treated embryos was not suitable for quantitative analysis. Antibodies that identify neurons and glial cells immediately after differentiation are available (see N. J. Messenger and Warner, 1989). Unfortunately the combination of the inherent variability of antibody staining, the requirement for serial, frozen sections through individual embryos and variation from embryo to embryo renders quantitative immunocytochemistry impracticable.

The results can be encompassed within a single hypothesis: that endogenous noradrenaline activates -adrenergic receptors as part of the control of neuronal differentiation

from the neural plate. The key findings are that treating neurulating embryos either with inhibitors of noradrenaline biosynthetic enzymes or with agents that block -adrenergic receptors is sufficient to prevent subsequent neuronal differentiation, whether assayed in culture or in vivo.

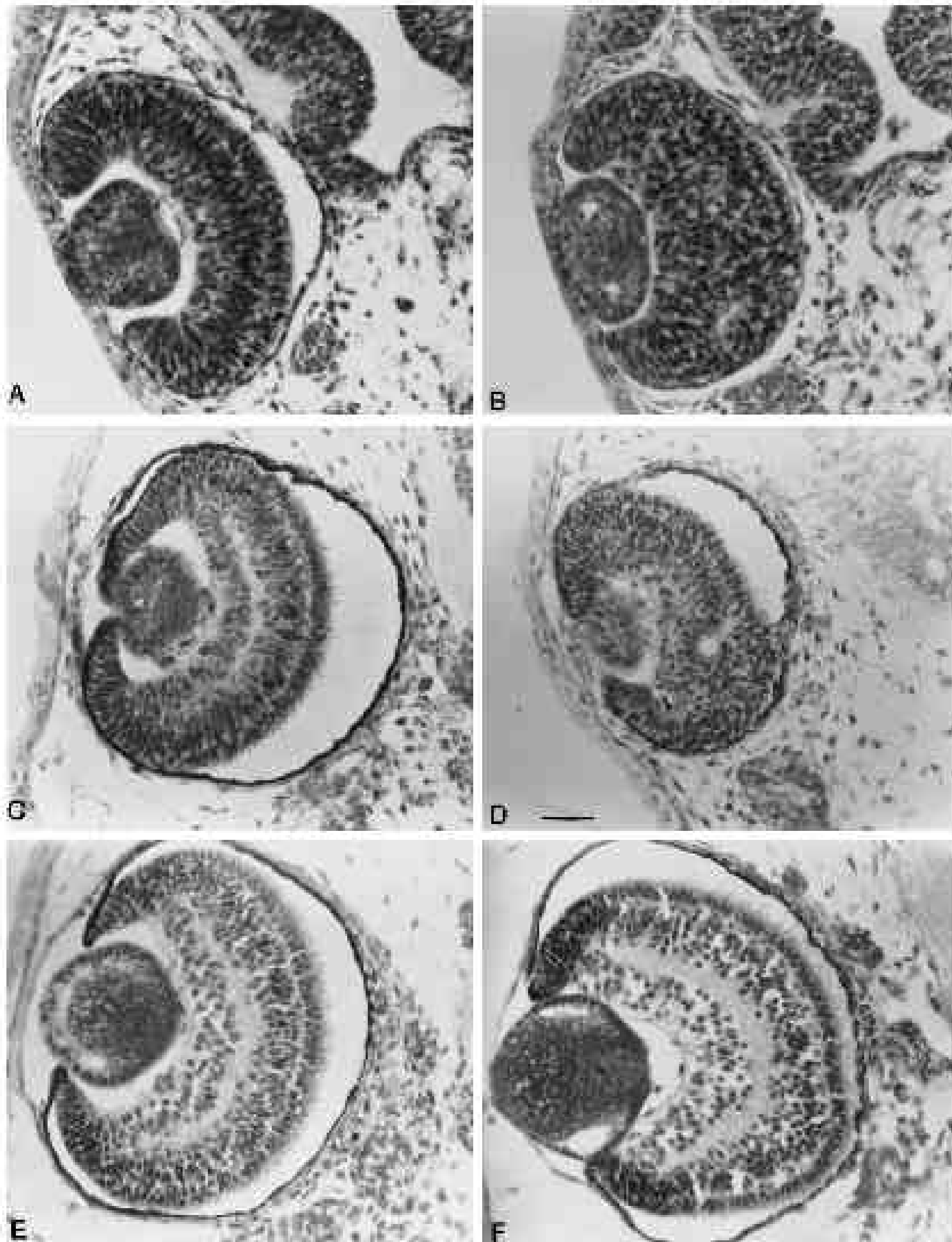

Fig. 9. CNS defects in intact tadpoles after treatment during neurulation with reagents that inhibit neuronal differentiation. Embryos were treated from stage 14 to 20. After washing out the drugs, the embryos were allowed to develop to stage 38 (A-D) or stage 40 (E,F) before sectioning. Each photograph shows a section through the eye. Bar, 50 μm. Controls are shown (A,C,E). In B (treated with 2×10^{-6} M diethyldithiocarbamate, an inhibitor of dopamine -hydroxylase) and D (treated with 10^{-8} M desipramine), the ordered layers of the neural retina are disrupted and the white matter in the brain is reduced. The effects are similar for both drugs, despite their different modes of action. Note that the -adrenergic receptor blocker propranolol (10^{-6} M: F) had no effect in vivo. The embryos shown in E and F are slightly older so the eyes are larger and development is more advanced.

Noradrenaline is the endogenous activating monoamine

Evidence for the involvement of endogenous noradrenaline in expression of the neuronal phenotype came from experiments with inhibitors of dopamine -hydroxylase, which catalyses the conversion of dopamine to noradrenaline, and caused substantial inhibition of the number of neurons differentiating in culture without influencing myocyte differentiation or overall differentation. The effect was opposed by exogenous noradrenaline. The development of the intact CNS was disturbed also. It is likely that endogenous noradrenaline is synthesized de novo from a dopamine pool since noradrenaline was detected at low concentration, only during the neural plate stages and the concentration of dopamine changed little during neurulation. Inhibitors of dopamine -hydroxylase prevented also exogenous dopamine from overcoming the consequences of -receptor blockade, but were ineffective in the presence of noradrenaline. This argues against an effect apart from that on noradrenaline synthesis.

Preventing noradrenaline synthesis with dopamine -hydroxylase inhibitors always reduced significantly neuronal differentiation. By contrast, exogenous noradrenaline, its analogues and dopamine were only variably effective (20% of trials) in promoting the differentiation of neurons, although when effective they provoked a very substantial potentiation. Furthermore, when dopamine, noradrenaline and agonists at -adrenergic receptors opposed the effects of -receptor blockers, agonist-induced potentiation of neuronal differentiation above controls was not unusual. It is impossible to know how much noradrenaline is needed to achieve the maximal differentiation of neurons. The safety factor could be relatively high, since the primary differentiation of neurons is a crucial event, in which case endogenously synthesized noradrenaline ought normally to be sufficient. Failure always to promote neuronal differentiation with exogenous monoamines is therefore not so surprising as it might seem.

The lack of effect on neuronal differentiation of dopa decarboxylase inhibitors is not unexpected, since stored dopamine should be adequate to generate the requisite noradrenaline. However, the finding that dopa decarboxylase inhibitors prevented L-dopa from opposing -receptor blockade implies that dopa decarboxylases are available to convert L-dopa to dopamine. In normal development, conversion of endogenous L-dopa to dopamine may not be rate-limiting for noradrenaline synthesis. The rate-limiting step may be conversion of endogenous dopamine by endogenous dopamine -hydroxylase.

Noradrenaline acts through α-adrenergic receptors

Treatment of *Xenopus* embryos during neurulation with drugs defined as -adrenergic receptor antagonists, but not antagonists at other receptors, prevented subsequent neuronal differentiation. The effects were overcome by the simultaneous inclusion of noradrenaline or other -adrenergic receptor agonists. The reduction of neurons differentiating in tissue culture was paralleled in the intact nervous system.

There is no evidence that the test reagents were toxic. All are used widely on differentiated systems, both in vivo and in vitro, and many are in routine clinical use. There was no reduction in the differentiation of myocytes or in total cell numbers. None of the reagents affected differentiation when present only during the period between plating and assay. Finally, embryos left to develop after washout of the reagents showed no necrosis or cell death.

The ability to act as -adrenergic receptor antagonists links the compounds found to prevent neuronal differentiation. All were effective at concentrations appropriate for their known receptor blocking activity. There were no exceptions to this rule. Conversely, reagents known to bind to -adrenergic receptors, dopamine receptors, acetylcholine receptors, or able to act only on uptake mechanisms, were without influence on neuronal differentiation and development of the CNS was undisturbed. There does not seem to be any reported action other than -adrenergic receptor blockade that is common to all compounds found to act on neuronal differentiation. One could fall back on some unidentified property, but it is more likely that blockade of -adrenergic receptors underlies the reduction in neuronal differentiation.

The agonists able to overcome -adrenergic receptor blockade fit this view. Of the monoamines present in neurulating embryos, only noradrenaline was effective. Specific -adrenergic receptor agonists competed efficiently with -adrenergic receptor blockers, while an agonist specific for -adrenergic receptors did not.

The relative sensitivity to antagonists of the putative -adrenergic receptor did not match any of the receptor subtypes so far described (see Ruffolo et al., 1991), which means that receptor subtype cannot be used to implicate particular downstream pathways. The neural plate cell receptor may be an embryonic form.

What is the origin of the neurons (about 35%) that differentiate despite the presence of maximally effective concentrations of, for example, desipramine? 30-35% of neurons remained resistant to the sodium pump inhibitor strophanthidin also (E. A. Messenger and Warner, 1979). These authors showed that the strophanthidin-resistant population was probably derived from the neural crest and concluded that strophanthidin affected preferentially neural plate-derived neurons. It is likely that this is the case also for neurons resistant to -adrenergic receptor blockade. Embryos treated with -adrenergic receptor blockers showed no obvious reduction in melanophores, a neural crest derivative.

CNS defects are revealed in the intact nervous system

It is important that all reagents found to prevent neuronal differentiation in culture had parallel effects in the nervous system. These were characterized by the loss of the organization of cells in the developing neural tube and retina and reductions in the white matter, which fits a reduction in CNS neurons. The absence of necrotic cells and the maintenance of grey matter volume speak against loss of cells through death or reduction in cell division. The reduction in cells expressing the neuronal phenotype was not accompanied by

a major change in overall patterning of the nervous system. Embryos with almost no neurite outgrowth contained brain structures in their usual relative position along the antero-posterior axis. The hierarchical order in which neurons express their specific fate is not known, but these observations suggest that the decision to be (or not be) a neuron may precede decisions about specific subsets of neuronal phenotype.

The fate of cells that do not differentiate into neurons is not known; they could remain undifferentiated, arrest as neuroblasts or follow an alternative such as a glial fate.

Does noradrenaline activate the sodium pump?

The evidence supports the hypothesis that activation of a neural plate -adrenergic receptor by noradrenaline is part of the processes leading to the emergence of neurons from the neural plate. Monoamines influence the activity of the sodium pump (reviewed in Phillis and Wu, 1981) and the activation of sodium pumps in neural plate cell membranes is an integral part of the mechanisms controlling neuronal differentiation. Might noradrenaline activate the sodium pump in neural plate cells? It is sufficient either to block the sodium pump or to interfere with the noradrenaline system to prevent CNS neuron differentiation and the developmental outcome is identical. However, neural plate cells are not sensitive to sodium pump inhibition after the mid-neural fold stages, while -adrenergic receptor blockade was still effective at the late neural fold stage. This is consistent with the -adrenergic receptor mechanism being downstream to sodium pump activation. However resolution of this issue requires exploration of the inter-relation between sodium pump activation and -adrenergic receptor activation (Rowe and Warner, unpublished data).

Conclusions

The results suggest that activation of an -adrenergic receptor by noradrenaline is an integral part of the mechanisms controlling the emergence of CNS neurons from the neural plate. This process may operate in sequence or in parallel with activation of the sodium pump; inhibition of either pathway is sufficient to prevent the successful differentiation of CNS neurons. Whether the monoamine-activated pathway is part of permissive or instructive signalling during specification of the neuronal phenotype remains to be elucidated. It will be interesting to see how the mechanisms considered here might interact with recently described local inductions such as those emanating from the notochord and floor plate (e.g. Placzek et al., 1993).

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