Int. J. Dev. Biol. 45: 767-770 (2001)

Short Communication

The neural inductive signal is transferred to ectoblast in 1-2 h but a continued contact with mesoblast for 2-3 h is essential for neuralization in the chick area pellucida

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ABSTRACT In the area pellucida of the chick gastrula, the Hensen's node (HN) graft must contact the competent ectoblast for at least 4 h to promote neural induction. When we removed the grafted HN after 1 to 3 h and replaced it by a non-inducing post nodal (PN) fragment, a 1-2 h contact with HN was found to be sufficient to promote neural induction. When HN graft was removed after 3 or 4 h and replaced by PN, the neural inductive response was substantially improved towards formation of archencephalic structures. Thus, our results indicate that neural induction takes place in two steps. In the first step, a contact with HN for 1-2 h is sufficient to transfer the inductive signal which is stabilized through a second step involving continued cell-cell contact with even non-inducing PN mesoblast.

KEY WORDS: *Neural induction, signal transfer, signal stabilization, Hensen's node, post-nodal fragment*

In chick gastrula the Hensen's node (HN) acts as the Organizer (Waddington, 1932) and the neural inducing property is restricted to the region surrounding the anterior end of the primitive streak (Mulherkar, 1958; Gallera and Ivanov, 1964; Gallera, 1971). According to Waddington, (1952), the neural induction occurs in two steps namely, *evocation* when the neural phenotype is determined in the ectoderm, and *individuation* when the neural tube acquires regional specificity. In this hypothesis, the two steps are spread over the entire period spanning gastrulation and neurulation. During neural induction, the inducer HN and the competent ectoblast establish a close contact (Gallera, 1971; England and Cowper, 1976) and the intensity of the inductive response depends on the duration of the contact (Gallera, 1971). A minimum contact period of 4h is required to induce area pellucida ectoblast while at least 8h-contact is essential in the area opaca (Gallera, 1965, 1970, 1971; Leikola, 1976; Storey *et al.,* 1992). It is not clear when the process of neural induction begins (Dixon and Kintner, 1989; Streit and Stern, 1999), nor is the precise chronology of cellular and molecular events during the inductive signal transfer known. Here, we show that the early manifestation of neural induction occurs in two time-bound steps. The inductive signal transfer from HN to the competent ectoblast takes at least 1h but the neuralization is detected only if the contact is maintained with at least noninducing postnodal fragment.

The HN was grafted on area pellucida ectoblast of a stage-4 chick embryo. The graft was removed after remaining in contact for 1 to 4h and replaced with an equivalent size PN (Fig. 1). When HN was left for 2 or 4h, the host ectoblast did not exhibit neural response (Table I). After a contact for 4h, only 1 out of 5 embryos exhibited induced neural tube (Fig. 2A, Table I). When HN was removed after a contact for 1h and replaced by PN, a palisade induction was observed (Fig. 3 B,C). The replacement by PN of HN contacted for 2 or 3h (Fig. 3 D-H) increased the amount of induced neural tissue, which exhibited regionalisation (Table I). With HN contact for 4h, followed by PN, induction of a complete neural tube was obtained with regionalisation (Fig. 3 I-K). Control PN graft does not induce neuralization (Fig. 3A).

We show that, except for graft-derived endoblast (Fig. 4A), ^{3}H-TdR labeled PN cells, replacing unlabeled HN, spread away from the grafting site and join the host extra-embryonic mesoblast (Fig. 4B) in the area vasculosa, coelom, lateral plates and the cardiac primordia. Thus, the induced ectoblast does not influence the migratory behavior of non-inducing PN mesoblast after the second half of the contact period.

It was suggested that neural induction may involve two distinct but spatially (Nieuwkoop, 1952; Gallera, 1971; Doniach, 1993), and chronologically (Dixon and Kintner, 1989; and present data) segregated signals. In the chick gastrula, a continuous contact of

Abbreviations used in this paper: HN, Hensen's node; PN, Post-nodal piece; ^{3}H-TdR, Tritiated thymidine.

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0214-6282/2001/$25.00

Printed in Spain
www.ijdb.ehu.es

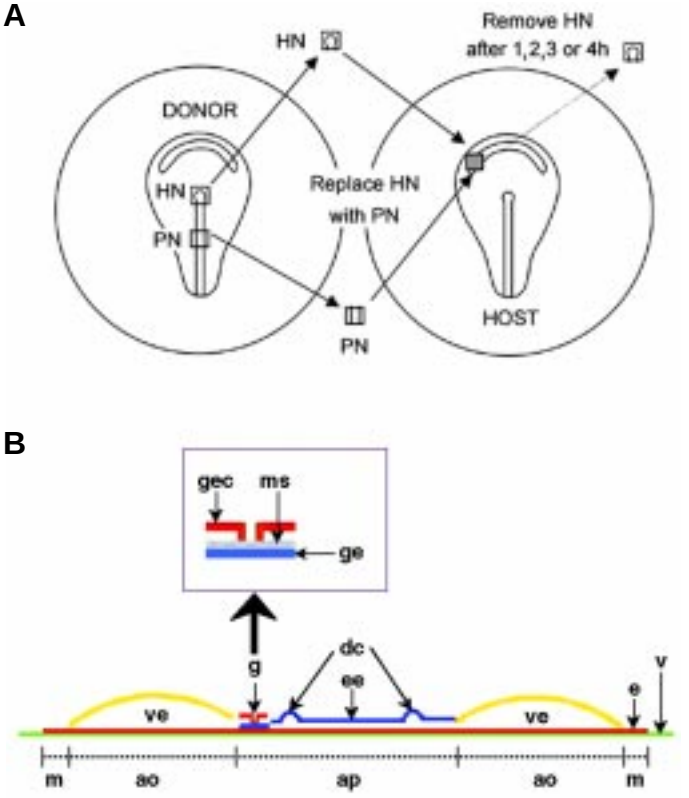

Fig. 1. Schematic drawing of the experimental design. (A) *Hensen's node (0.2 mm x 0.2 mm) was extirpated and transplanted on stage 4 host blastoderm at 10 o' clock position. After 1, 2, 3 or 4 h culture, the HN graft was scraped off and replaced by a PN graft (0.2 mm x 0.2 mm) and the culture was continued till 20 h* in vitro. **(B)** *Transverse section of the host embryo receiving a graft,* g*, as described by Gallera (1971). The graft is placed between the anterior rim of the area pellucida* (ap) *and the Duval crescent* (dc) *with the graft-endoblast* (ge) *in contact with host ectoblast* (e). *ms, mesoblast; v, vitelline membrane; ve, vitelline endoderm; ao, area opaca; m, margin of overgrowth; ee, host embryonic endoblast; gec, graft ectoblast.*

at least 4h between ectopically grafted HN and the competent ectoblast of the area pellucida is essential to obtain a neural induction (Gallera, 1965, 1970, 1971; Storey *et al.,* 1992) and we (Joshi, 1999 and present data) confirm these studies. Furthermore, the contact period appears to be made up of two distinct time

TABLE I

ANALYSIS OF NEURAL INDUCTION OBTAINED BY REPLACING HENSEN'S NODE BY POSTNODAL PIECE

Hours of contact		Number of	Neural	Type of Neural Induction*			
HN	PN	experiments	inductions	A	B	C	D
2	0	5	0	-	-	-	-
4	0	5	1	-	-	1	-
6	0	4	4	-	-	3	1
9	0	4	4	-	-	1	3
20	0	5	5	-	-	2	3
0	20	5	0	-	-	-	-
1	19	6	5	4	1	-	-
2	18	9	9	3	6	-	-
3	17	5	5	1	4	-	-
4	16	13	13	1	2	10	-

*A, Palisade – induced tissue consisting of elongated cells set closely side-by-side. B, Medullary – pseudostratified columnar cells forming a thickened trough-like neural plate with a depression at the center. C, Archencephalic – containing anterior region of the neural tube including the fore brain in the absence of notochord. D, Deuterencephalic – containing posterior brain and the spinal cord.

steps (Joshi, 1999 and present data); the first requires an obligatory contact with the inducer HN for at least 1h, while in the second step, the ectoblast having already reacted with HN for 1h should be allowed to contact at least noninducing tissue to sustain neuralization. We suggest that the inductive signal is transferred from the HN during the initial period of contact but its stabilization leading to the manifestation of the neural response requires a continued cell-cell contact with at least non-inducing cells for another 2h. As HN and PN contain presumptive dorsal and posterior mesoblast, respectively, a continued contact with mesoblast appears to be essential to stabilize the inductive process. Electron microscopy has revealed that a single mesoblast cell may contact many ectoblast cells during neural induction (England and Cowper, 1976). Furthermore, even during the so-called trans-filter induction, cytoplasmic processes invade the filter and contact the reactive ectoblast (Gallera *et al.,* 1968). During normal development the inductive signal probably continues to be transferred beyond first 1-2h. Indeed, a continued flow of the signal for longer periods may allow individuation in the induced ectoblast. This is consistent with our observation that by increasing the duration of contact with HN from 1 to 4h, followed by replacement with PN, the nature of the induction shifts progressively from the primitive palisade to medullary to archencephalic type. Thus, we show for the first time that different duration of exposure to each of the two signals results in a different rostro-caudal level of neural induction.

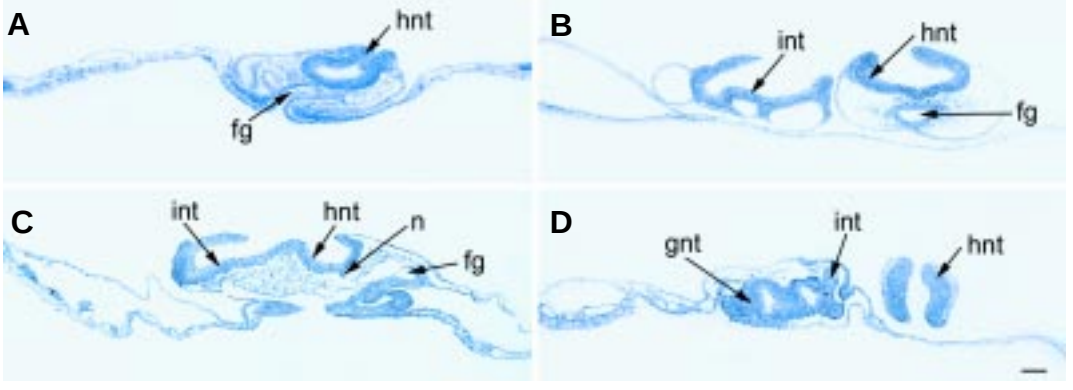

Fig. 2. Neural response to HN-contact observed in host ectoblast. *Sections of host embryo with HN graft left in contact for* **(A)** *4 h,* **(B)** *6 h and* **(C)** *9 h followed by culture till 20 h, and* **(D)** *20h. Neural induction is observed in 1 out of 5 cases after a contact of 4 h. With HN-contact longer than 4 h, neural tissue is always induced. hnt, host neural tube; int, induced neural tube; fg, foregut; n, notochord; gnt, graft neural tube. Scale bar, 100 μm.*

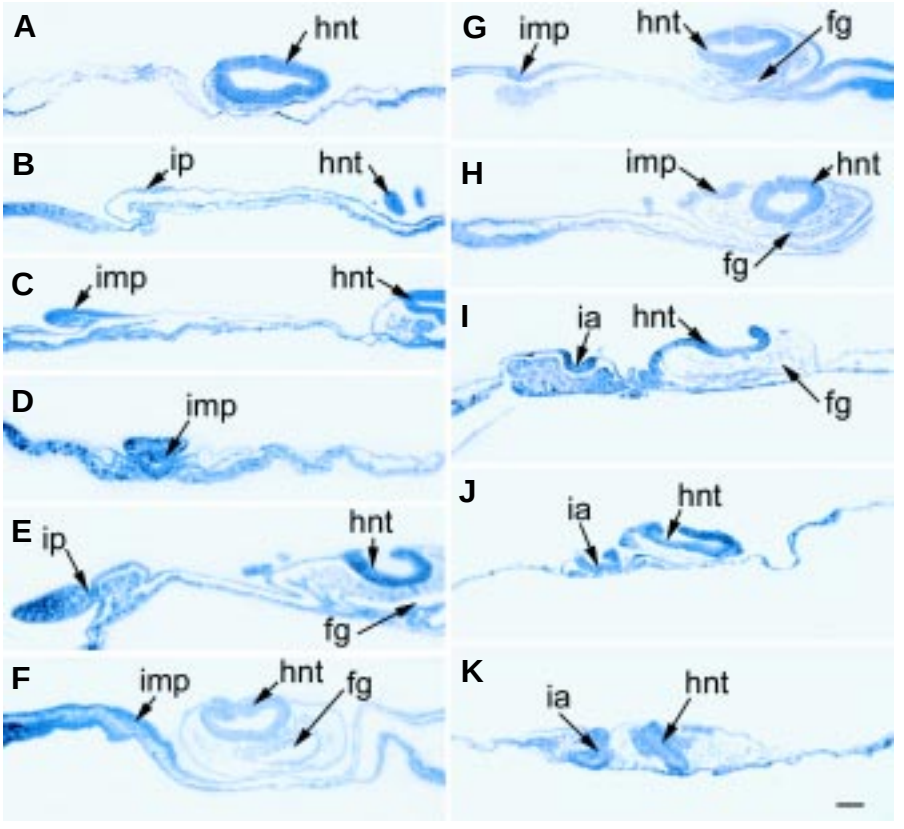

Fig. 3. Neural response observed when HN is replaced by PN after varying hours of contact. *Transverse section with* **(A)** *PN alone 20 h,* **(B,C)** *HN 1 h and PN 19 h,* **(D,E)** *2 h HN and 18 h PN,* **(F-H)** *3 h HN and 17 h PN,* **(I-K)** *4 h HN and 16 h PN. These show increasing size and differentiation of the induced neural tissue with longer time of contact with HN. ip, induced palisade; imp, induced medullary plate; ia, induced archencephalon; hnt, host neural tube and fg, foregut. Scale bar, 100 μm.*

The secreted protein Noggin has been suggested to be the best endogenous factor for neural induction (Lamb *et al.,* 1993; Yamada, 1995). bFGF induces neural tube and neural crest lineages of cultured ectoblast cells from *Xenopus* gastrula (Kenagaku and Okamoto, 1993) and *Triturus* ectoderm explants (Tiedemann *et al.,* 1994). In chick, FGF-coated heparin acrylic beads transplanted at ectopic sites induce morphologically recognizable neural tissue within 1-2h of contact (Rodriguez-Gallardo *et al.,* 1997; Alvarez *et al.,* 1998) and induce expression of the early neural marker *ERNI* similar to that with HN graft (Streit *et al.,* 2000) or posterior neural markers *Sax-1* and *cash4* (Storey *et al.,* 1998). In this context, our results (Joshi, 1999; present data) give credence to the possibility that induction is a multi-step process and FGF and Noggin appear to be among the essential components of the initiation process to sensitize the epiblast to the downstream events (Kengaku and Okamoto, 1993; Lamb *et al.,* 1993; Tiedemann, *et al.,* 1994; Foley *et al.,* 2000; Stern *et al.,* 2000). *Chordin* is presumably located downstream of the initial neuralizing signal (Streit *et al.,* 1998) and stabilizes the induced state of cells. However, it is unlikely that a complex process of neuralization and region-specific differentiation depends on only one or two marker genes, an issue that needs to be resolved by studying the entire sequence of expression of other marker genes in our experimental model.

It is known (Modak, 1966; Nicolet, 1970) that ^{3}H-TdR labeled HN graft self-differentiates into a neural plate, notochord, somites and small amount of definitive endoblast. Similarly, labeled PN give rise to cardiac, paraxial as well as extra-embryonic mesoblast including blood islands (Joshi, *et al.,* unpublished). Furthermore, labeled HN graft cells do not move into the neural tissue induced in the host ectoblast. We now show (Fig. 3) that most ^{3}H-TdR labeled PN cells, replacing the inducer HN after 1h, move away from the graft site and only labeled graft-endoblast remains close to the induced medullary tube. We conclude that re-established cell-cell contact may lead to a selective retention of PN-endoblast while mesoblast cells spread out. The significance of this observation is not clear because only anterior definitive endoblast, produced during gastrulation (Modak, 1966) is known to have the inducing capacity (Gallera and Nicolet, 1969). Our findings open avenues for exploring whether the early period of neural induction involves novel cell surface receptor-ligand interactions.

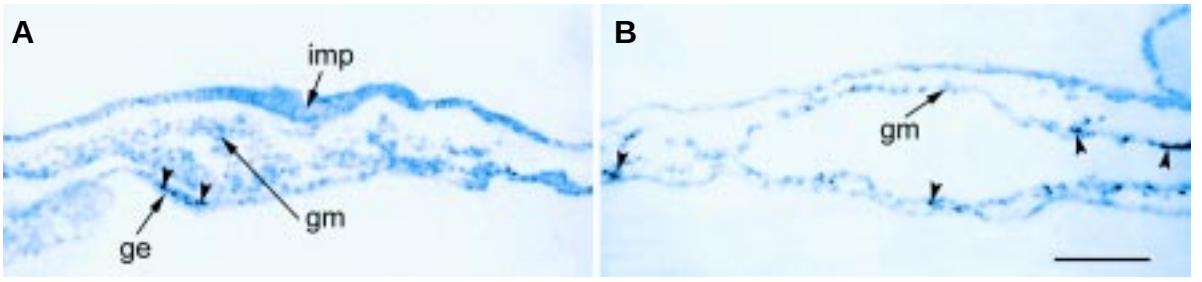

Fig. 4. Migratory behavior of grafted PN cells in the host embryo. *The HN graft was transplanted on a stage 4 host and then removed after a contact for 1 h and replaced by a ^{3}H-TdR-labeled PN graft. Embryos were fixed after culture for 20 h. Sections were coated with NTB2 (Kodak) liquid emulsion for autoradiography. ^{3}H-TdR-labeled cells are localized (arrow head) in* **(A)** *the graft endoblast (ge), underlying the induced medullary plate (imp) and in* **(B)** *graft derived mesoblast,(gm). Scale bar, 100 μm.*

Experimental Procedures

White Leghorn chick embryos of developmental stage 4 (Hamburger and Hamilton, 1951) were cultured *in vitro* (New, 1955). HN piece (0.2 mm x 0.2 mm) was grafted at 10 o'clock position between the anterior rim of the area pellucida and the Duval's (germinal) crescent of another stage 4 host as shown in Fig. 1 (Gallera, 1971). Grafts were left in contact with the host ectoblast for 1,2,3,4 and 6 hours and then carefully scraped off with an irido-platinum wire loop. After removing the graft, PN fragment (0.2 mm x 0.2 mm), derived 0.7 mm posterior to the donor Hensen's node, was transplanted and pressed with its endoblast against the host ectoblast at the original HN graft site. The host embryos were cultured for a total duration of 20 h. Control hosts received HN alone for 1-6 h. In another control, PN was grafted on a stage 4 host and cultured for 20 h. At the end of the experiment, blastoderms were fixed (4 h) in ice-cold Carnoy containing ethanol, chloroform and acetic acid [6:3:1] for whole mounts or histology. Sections were stained in Mayer's haematoxylin, dehydrated and mounted in DPX.

To examine the behavior of the PN cells replacing the HN, donor embryo was labeled with ^{3}H-TdR (Sp. act. 12 Ci/mmole; 1 μCi / ml) for 1h. From the labeled embryo, the PN fragment was extirpated and transplanted in place of the HN, which had been removed after being in contact with the host ectoblast for 1-4 h. Embryos were cultured for a total duration of 20 h, fixed in Carnoy and embedded in paraffin. Deparaffinized serial sections were coated with liquid emulsion NTB3 (Kodak), exposed in dark for 4 days, developed in D-19, fixed and nuclei were stained as before.

Acknowlwdgements

This work was generously supported by the UGC & BRNS-DAE to SPM. DJB was a recipient of CSIR-Senior Research Fellowship, SD was recepient of a Lady Tata Memorial Fellowship. We thank Mahendra Sonawane and Srikanth Mathur for help in the laboratory. We are grateful to late Prof. Tuneo Yamada for critical comments.

References

ALVAREZ, I., ARAUJO, M. and NEITO, A. (1998). Neural induction in whole chick embryo cultures by FGF. *Dev. Biol.* 199: 42-54.

DIXON, J.E. and KINTNER, C.R. (1989). Cellular contacts required for neural induction in *Xenopus* embryo: evidence for two signals. *Development* 106: 749-757.

DONIACH, T. (1993). Planer and vertical inductions of antero-posterior pattern during the development of the amphibian central nervous system. *J. Neurobiol.* 24: 1256-1275.

ENGLAND, M.A. and COWPER, S.V. (1976). A transmission and scanning electron microscope study of primary neural induction. *Experientia* 32:1578-1580.

FOLEY, A., SKROMNE, I. and STERN, C.D. (2000). Reconciling different models of forebrain induction and patterning: a dual role for the hypoblast. *Development* 127: 3839-3854.

GALLERA, J. (1965). Quelle est la durée necessaire pour déclencher des inductions neurales chez le poulet. *Experentia* 21: 218.

GALLERA, J. (1970). Difference in reactivity to the neurogenic inductor between the ectoblast of the area opaca and that of the area pellucida in chickens. *Experientia* 26:12, 1353-1354.

GALLERA, J. (1971). Primary embryonic induction in birds. *Adv. Morphogen.* 9: 149-180.

GALLERA, J. and IVANOV, I. (1964). La compétence neurogène du feuillet externe du blastoderm de poulet en fonction du facteur 'temps'. *J. Embryol. Exp. Morph.* 12: 693-711.

GALLERA, J. and NICOLET, G. (1969). Le pouvoir inducteur de l'endoblaste présomptif contenu dans la ligne primitive jeune de poulet. *J. Embryol. Exp. Morph.* 21: 105-118.

GALLERA, J. NICOLET, G. and BAUMANN, M. (1968). Induction neurale chez les oiseaux à travers un filtre millipore: étude au microscope optique et électronique. *J. Embryol. Exp. Morph.* 90: 439-450.

HAMBURGER, V. and HAMILTON, H.L. (1951). A series of normal stages in the development of the chick embryo. *J. Morph.* 88: 49-92.

JOSHI, D. (1999). The role of presumptive posterior mesoderm in morphogenesis and cell population growth of chick embryos. Ph.D. Thesis. University of Pune, Pune, India, pp. 1-68.

KENGAKU, M. and OKAMOTO, H. (1993). Basic fibroblast growth factor induces differentiations of neural tube and neural crest lineages of cultured ectodermal cells from *Xenopus* gastrula. *Development* 119: 1067-1078.

LAMB, T.M., KNECHT, A.K., SMITH, W.C., STACHEL, S.E., ECONOMIDES, A. N., STAHL, N., YANCOPOULOSE, G.D. and HARLAND, R.M. (1993). Neural induction by secreted polypeptide Noggin. *Science* 262: 713-718.

LEIKOLA, A. (1976). Hensen's node -The 'Organizer' of the amniote embryo. *Experientia* 32: 269-277.

MODAK, S.P. (1966). l'Analyse expérimentale de l'origine de l'endoblaste embryonnaire chez les oiseaux. *Rev. Suisse Zool.* 73: 877-908.

MULHERKAR, L. (1958). Induction by regions laterals to the streak in the chick embryo. *J. Embryol. Exp. Morph.* 6: 1-14.

NEW, D.A.T. (1955). A technique for cultivation of chick embryo *in vitro. J. Embryol. Exp. Morph.* 3: 326-331.

NIEUWKOOP, P.D. (1952). Activation and organization of the central nervous system in amphibians. III Synthesis of a new working hypothesis. *J. Exp. Zool.*, 120: 83-102.

NICOLET, G. (1970). Analyse autoradiographique de la localisation des differentes ébauches présomptives dans la ligne primitive de l'embryon de poulet. *J. Embryol. Exp. Morph.* 23: 79-108.

RODRIGUEZ-GALLARDO, L., CLIMENT, V., GARCIA-MARTINEZ, V., SCHOENWOLF, G. and ALVAREZ, I. (1997). Targeted over-expression of FGF in chick embryos induces formation of ectopic neural cells. *Int. J. Dev. Biol.* 41: 715-723.

STOREY, K.G., CROSSLEY, J.M., DE ROBERTIS, E.M., NORRIS, W.E. and STERN. C.D. (1992). Neural induction and regionalisation in the chick embryo. *Development* 114: 729-741.

STOREY, K.G., GORIELY, A., SARGENT, C.M., BROWN, J.M., BURNS, H. D., ABUD, H.M. and HEATH, J.K. (1998). Early posterior neural tissue is induced by FGF in the chick embryo. *Development* 125: 473-484.

STREIT, A., LEE, K., WOO, I., ROBERTS, C., JESSEL, T.M. and STERN, C. (1998). Chordin regulates primitive streak development and the stability of induced neural cells, but is not sufficient for neural induction in the chick embryo. *Development* 125: 507-519.

STREIT, A. and STERN, C.D. (1999). Neural Induction-a bird's eye view. *Trends in Gen.* 15: 20-24.

STREIT, A., BERLINER, A.J., PAPANAYOTOU, C., SIRUINIK, A. and STERN, C.D. (2000). Initiation of neural induction by FGF signaling before gastrulation. *Nature* 406: 74-78.

TIEDEMANN, H., GRUNZ, H., LOPPNOW-BLINDE, R. and TIEDEMANN, H. (1994). Basic fibroblast growth factor can induce exclusively neural tissue in *Triturus* ectoderm explants. *Roux's Arch. Develop. Biol.* 203: 304-309.

WADDINGTON, C.H. (1932). Experiments of chick and duck embryos cultivated *in vitro. Phil. Trans. Roy. Soc.* B 122: 171-179.

WADDINGTON, C.H. (1952). The Epigenetics of Birds. *Cambridge University Press*, London.

YAMADA, T. (1995). Anteroposterior specification in amphibian embryogenesis: The regulative roles of positive and negative controls of mitogenesis. *Dev. Growth Differ.* 37: 365-372.

Received: November 2000
Reviewed by Referees: January 2001
Modified by Authors and Accepted for Publication: May 2001