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Original Article

The neural inductive response of competent chick ectoblast decreases away from the host axis and correlates with an increased proliferative activity

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ABSTRACT We have assessed the quality and quantity of the neural inductive response of the chick gastrula ectoblast located at increasing distance from the host axis. In a stage 4 gastrula, entire ectoblast exhibits neural competence. The quality of induced neural tissue shifts from deuterencephalic type in the area pellucida to archencephalic type in the area opaca and primitive medullary or palisade type at the margin of overgrowth with a concomitant reduction in the number of induced neural cells. In contrast, the mitotic and ^{3}H-TdR labelling frequencies in the competent ectoblast increase with increasing distance from the host axis and in a proportion inverse to the amount of induced neural tissue. It is suggested that the strong neural inductive response is correlated with low proliferative activity, or longer cell cycle time, of the competent ectoblast.

KEY WORDS: *neural competence, induction, cell proliferation, chick embryo*

Introduction

The induction of vertebrate nervous system requires a vertical interaction between competent ectoblast and inducer chordamesoblast (Spemann and Mangold, 1924; Waddington, 1930; 1932). Another possibility, considered by Spemann (1938), that the neural induction may initiate via horizontal or planar flow of the inducer originating at the dorsal lip received experimental support (Nieuwkoop, 1952 and Doniac, 1993). Considering that the ectoblast is a flat contiguous sheet of cells in the flat discoid chick embryo, Gallera (1971) resurrected the horizontal planar flow model for the inductive stimulus. This model has been revived recently for amphibians (Sharp *et al.*, 1987; Doniach *et al.*, 1992; Keller *et al.,* 1992; Ruiz I Altaba *et al.*, 1992; Doniach, 1993; Pera and Kessel, 1997).

In amphibians (Lehman, 1929; Mangold, 1933; Holtfreter, 1938a,b; Nieuwkoop, 1958; Johnen, 1956a,b; 1961; Gallera, 1947; 1952 and 1959) and birds the neural competence diminishes (Woodside, 1937) and disappears abruptly after gastrulation (Gallera and Ivanov, 1964; Gallera, 1971). In the chick, the neural competence is regulated temporally (Waddington, 1930, 1932; Woodside, 1937; Gallera and Ivanov, 1964) and spatially (Gallera and Ivanov, 1964; Gallera, 1971; Leikola, 1976; Storey *et al.*, 1992). In the flat discoid chick embryo the neural inductive response in the contiguous sheet of ectoblast is better in the area pellucida than the area opaca (Gallera and Ivanov, 1964; Gallera, 1965; Leikola and McCallion, 1967; Gallera, 1971; Leikola, 1976). For Nieuwkoop (1952), amphibians exhibit 2 distinct periods of competence for anterior (archencephalic) and posterior (deuterencephalic) inductions. Varying combinations of neuralizing and mesodermalizing factors probably generate these differential effects (Chuang, 1940; Toivonen, 1940; Saxen and Toivonen, 1962) transmitted by chordamesoderm during involution and convergent extension (Vogt, 1929; Yamada, 1950; 1994; 1995).

Changing the duration of cell-cell contact affects the nature of neural inductive response (Gallera, 1971). Dissociation and aggregation changes the commitment of competent ectoblast towards formation of the neural cell lineage and thus regulate the neural fate (Serventrick and Grainger, 1991). Examination of amphibian ectoblast by polarising microscopy revealed (Gallera and Baud, 1954) that the subcellular channels change their orientation with a loss of neural competence. Moreover, treatment with urea prolonged the neural competence and retained the original channel orientation (Gallera and Baud, 1954). Chick gastrula ectoblast cells exhibit different morphology along the radial axis (Downie, 1976) and the proliferative activity is maximal at the margin of overgrowth, (New, 1959; Sonawane, 2000). Unfortunately, the possible relevance of these parameters to differences in the state of "competence" has never been given a serious thought.

Abbreviations used in this paper: HN, Hensen´s Node; ^{3}H-TdR, tritiated thymidine.

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Here we assess the intensity of neural competence from the quality and quantity of neural tissue induced by the Hensen's node. We find that grafting HN increasingly away from the host axis shifts the quality of induced neural tissue from deuterencephalic type in the area pellucida to archencephalic type in the area opaca and to even more primitive medullary type at the margin of overgrowth, with a concomitant reduction in the induced neural cell number. In contrast, the proliferative activity of ectoblast increases away from the host axis.

Results

The Hensen's node (HN) was grafted on a stage 4 host (Fig. 1) in the area pellucida (position A), the area opaca (position B) or the margin of overgrowth (position C). At each position, 15 embryos received one graft and in all 45 such hosts were cultured for 20h, drawn to scale on the Camera Lucida and fixed. At each position, 5 whole mounts and all serially sections of 4-6 embryos were examined for cell numbers and morphometric parameters.

At all grafting sites, host ectoblast formed induced neural tissue. At positions A and B, each graft formed a mini embryonic axis, while the graft-derived axis was less distinct at position C. From morphology in sections the induced neural tissue is classified as neuroidal, palisade, medullary or a complete neural tube (Sherbet, 1963; Gallera, 1971). In neuroidal reaction host ectoblast is thickened with pseudostratified large round cells. Palisades involve thickened ectoblast with elongated columnar cells resembling a primitive neural placode / plate. In medullary type, the neural plate forms a shallow trough with dorso-laterally raised neural folds. Finally, a well-formed closed neural tube is present which undergoes regionalisation as archencephalic (forebrain), deuterencephalic (mid- and hind- brain) and spino-caudal (spinal cord) or the entire neural tube. In an archencephalic structure, the neural tube (forebrain) lacks underlying notochord, while the deuterencephalic (mid-hind brain) or spinocaudal structures are underlined by the notochord of graft origin.

Positional differences in the nature of the inductive response

At position A, the induced neural tube was deuterencephalic in 3 out of 5 hosts (Fig. 2 A,B) or archencephalic (Fig. 2C) in 2 hosts. At position B, the neural tube is archencephalic (Fig. 2 D,E) or medullary type (Fig. 2F); while at the margin of overgrowth palisade type (Fig. 2 G,H,I) of neural tissue is induced (Table 1). Thus, the quality of induction shifts from deuterencephalic to increasingly primitive neural structure as one move away from the host axis. Similarly, the induced neural tissue becomes progressively smaller from the position A to C.

Quantification of the induced neural tissue

The morphometry of serial sections reveals (Table 1) that induced tissue volume is 5.55×10^{-2} mm^3 in the area pellucida and decreases significantly ($p<0.05$) to 2.6×10^{-2} mm^3 in the area opaca and 1.54×10^{-2} mm^3 at the margin of overgrowth (Fig. 3A; Table 1). The induced cell number also decreases significantly ($p<0.05$) from 1.29×10^4 cells in the area pellucida to 0.61×10^4 in the area opaca and to 0.33×10^4 at the margin of overgrowth (Table1; Fig. 3B). The mitotic frequency in the induced neural tissue in the area pellucida is $7.34 \pm 2.9\%$ decreasing to $3.84 \pm 0.6\%$, at margin of overgrowth, but this is not statistically significant ($p>0.05$). At all positions the cell population density of the induced tissue is similar (Table 1). We conclude that quantity of induced neural tissue in the ectoblast decreases as HN is grafted at positions increasingly away from the host axis (Fig. 2 A-I).

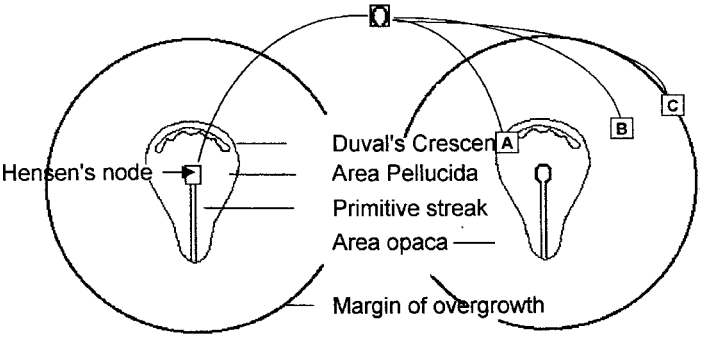

Fig. 1. A schematic diagram of Hensen's Node grafted on a stage 4 chick blastoderm. *Grafts are placed on* **(A)** *the area pellucida,* **(B)** *the area opaca, and* **(C)** *the margin of overgrowth.*

In serial sections, the induced neural tissue in host is contiguous with the host ectoblast whereas the graft-derived tissue is towards the endodermal side of the host (Fig. 2 C,F,G). Furthermore, grafting a ^{3}H-TdR-labelled HN clearly results in location of the graft-derived tissue ventrally, or towards the host endoderm, while the neural tissue induced dorsally in the host ectoblast is not labelled (Fig. 4). Therefore, it is possible to differentiate between the induced tissue and graft-derived tissue. At positions A and B, the graft differentiates into a neural tube, notochord, somites and gut endoderm in all 5 hosts (Table 2; Fig. 2 A-F). At position C, grafts in all 5 hosts form neural tube but in only a few show notochord and somite differentiation (Table 2, Fig. 2 G-I). The total volume of the graft-derived tissues decreases from 4.074×10^{-2} mm^3 in the area pellucida to 1.36×10^{-2} mm^3 at the margin of overgrowth (Fig. 3A; Table 2). The observed volume differences between positions B and C are statistically significant ($P<0.05$). Between the pair A and C the volumes are very weakly significant, if at all ($P<0.095$), while that between the pair A and B are not statistically significant.

Graft-derived neural cell number decreases significantly ($p<0.05$) from 0.91×10^4 in the area pellucida to 0.21×10^4 at the margin of overgrowth (Fig. 3B). The number of graft neural cells at position B and C are also statistically different ($p<0.05$) while the values for A and B are not. The situation is similar for the notochord (Table 2). Finally, the decreased mitotic frequencies in the graft neural tissue and mesoderm are not statistically significant certainly due to the small data size. The amount of graft-derived tissue is similar at positions A and B, and we find that the graft volume, neural cell number ($p<0.05$) and the mitotic frequency ($p<0.025$) are significantly greater than those at position C. Again, the graft cell population density is the same at all 3 positions (Table 2).

HN labelled with ^{3}H-TdR and grafted on unlabelled host, shows no label in the induced tissue (Fig. 4) indicating that graft cells do not migrate to and contribute for the induced tissue.

Cell proliferative activity: The mitotic frequency in the ectoblast disc was estimated from whole mounts and it is the lowest (2.9%) at the position A and increases to 4.36% at position B to 5.6% at position C. After 30min labelling, ^{3}H-TdR labelling frequency in whole mounts shows 1.2% labelled cells at position A increasing to 29.1%

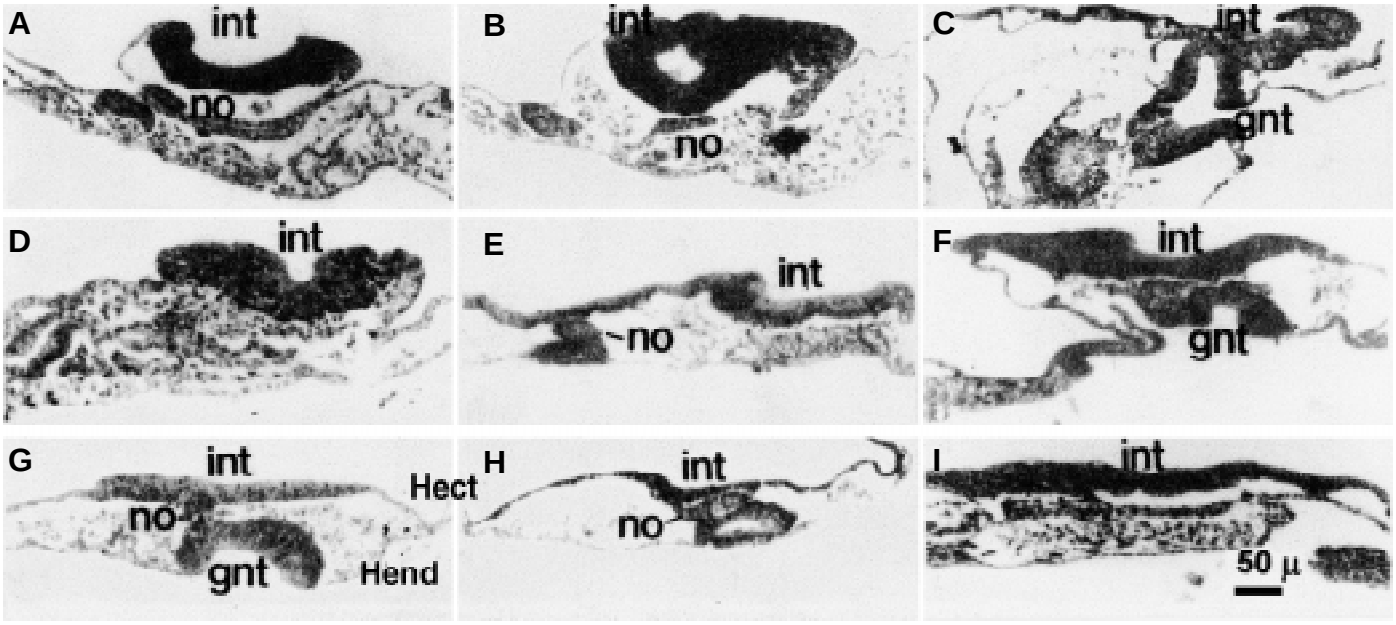

Fig. 2.Transverse sections of chick embryos transplanted with Hensen's node showing induced neural tissue (int), and graft-derived neural tissue (gnt) and notochord (no). *At position A, we observe* **(A,B)** *deuterencephalic and* **(C)** *archencephalic types of induction.* **(D-F)** *At position B, archencephalic types of induction can be seen.* **(G-I)** *At the position C, a palisade type of induced neural tissue is observed. Note that, as in (G), the induced tissue is contiguous with the host ectoblast (Hect) whereas graft derived tissue is towards the host endodermal side (Hend).*

at position C. When the labelling frequencies were determined from sections these are 16.5% at position A increasing to 39.2% at position B and 59% at the margin of overgrowth (position C) (Fig. 5 A-F, Table 3). Thus, the estimated labelling frequencies in the ectoblast decrease consistently as one moves towards the embryonic axis and the differences in labelling frequencies scored from whole mounts and sections are normal since the self-absorption coefficient for weak beta emitted by tritium would lead to low incidence on the photographic emulsion over whole mounts. Furthermore, differences in labelling frequencies at different positions are real (Table 3) and not artefacts produced by differences in diameters of nuclei falling within tritium-autoradiographic range. For the area pellucida, our estimated labelling frequency in whole mounts is lower than in sections (Table 3). From serial sections, the entire ectoblast region, anterior to Hensen's node, was reconstructed and the labelling frequencies in the five concentric zones were determined. We find that the labelling frequency increases exponentially away from HN towards the margin of overgrowth (Fig. 5 G).

Discussion

The Hensen's node, grafted on a stage 4 chick embryo, induces neural tissue in the ectoblast in both pellucid and opaque areas (Fig. 2 A-F) as before (Gallera and Ivanov, 1964; Gallera, 1971; Leikola and McCallion, 1967; Leikola, 1976; Storey *et al.*, 1992; present data). We now add that the ectoblast at margin of overgrowth is also competent to be neuralised (Fig. 2 G-I, Fig 3 A,B; Table 1). The induced tissue size in the area pellucida is larger than in the area opaca, confirming earlier reports (Gallera and Ivanov, 1964; Gallera, 1971; Leikola and McCallion, 1967; Leikola, 1976; Storey *et al.*, 1992). From morphometric measurements of the size and cell number, we now show (Fig. 2 G-I) that induced neural tissue is the smallest at the margin of overgrowth. The induced neural cells exhibit typical neuroblast morphology, but the type of induction shifts from deuterencephalic in area pellucida to archencephalic in area opaca and medullary or palisade at the margin of overgrowth (Fig. 2 A-I). At positions A and B, the graft differentiates the same extent with a neural tube, notochord, somites and gut endoderm (Fig. 2 A-F, Table 2) as before (Gallera, 1971; Dias and Schoenwolf, 1990; Selleck and Stern, 1991 and Storey *et al.*, 1992) but considerably reduced at position C or the margin of overgrowth (Fig. 2 G-I). In the first ever analysis, we find that, at all the sites the cell population density and ratios for number of "Induced ÷ Graft-derived" cells are similar. Thus, the graft and the induced tissue grow and develop co-ordinately. We also show that graft cells do not migrate to and contribute for induced neural tube (fig. 4).

TABLE 1

QUALITATIVE AND QUANTITATIVE ANALYSIS OF INDUCED NEURAL TISSUE IN CHICK ECTOBLAST

Graft position	Number of embryos	Type of induction* S	D	A	M	P	Induced neural tissue Volume ($x10^{-2}$ mm^3)	Cell no. $x\ 10^4$	% mitosis	Cell Population Density Cell no./ tissue volume
Area Pellucida (A)	5	-	3	2	-	-	5.55 ± 2.00	1.29 ± 0.34	7.34 ± 2.99	2.33 ± 0.24
Area Opaca (B)	5	-	-	3	2	-	2.60 ± 0.90	0.61± 0.16	3.83 ± 0.60	2.43 ± 0.47
Margin of overgrowth (C)	5	-	-	-	1	4	1.54 ± 0.53	0.33 ± 0.13	3.81± 0.38	2.20 ± 0.56

S, Spinocaudal; D, Deuterencephalic; A, Archencephalic; M, Medullary; P, Palisade.

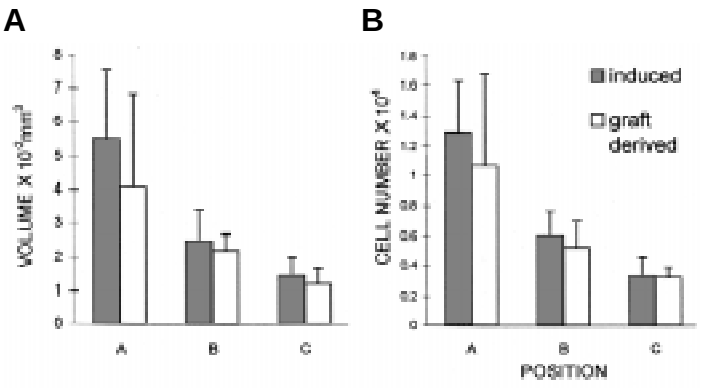

Fig. 3. Histogram of the induced and graft-derived neural (A) tissue volumes and (B) cell numbers in the area pellucida, the area opaca and the margin of overgrowth (Position C). *Note that both tissue volumes and cell numbers decrease from position A to B to C.*

Neural induction depends primarily on the ability of the host ectoblast to respond and that of the inducer to transfer the signal (Waddington, 1932). We consider 6 cellular and molecular parameters exhibiting time- and/or position-dependent changes in the ectoblast potentially relevant to the state of the endogenous reactive potential or neural competence towards the inductor. These are [a] cell morphology, [b] cell proliferation, [c] morphogenetic status [d] concentrations of morphogen/s acting as sensors, [e] the presence of "responder" molecules of defined half-life and [f] cell surface status.

The difference in the inductive response has been suggested (Gallera, 1971) as due to morphology of the host ectoblast. During gastrulation, area pellucida ectoblast undergo morphogenetic movements of convergence (see for review, Nicolet, 1971) while, at the margin of overgrowth, ectoblast undergo epiboly (New, 1959; Downie, 1976; Ghatpande *et al.*, 1991, 1993; Bellairs, 1986). thereby causing stretching of ectoblast sheet. Thus, the area pellucida ectoblast contains elongated pseudo-stratified epithelium with a small diameter as compared to cuboidal or flattened cells in the area opaca (Bellairs, 1959; Messier, 1969; Gallera, 1971; Downie, 1976; Ghatpande et al, 1993, present data). At the margin of overgrowth, ectoblast cells are highly irregular in shape and often anchored onto the vitelline membrane (New, 1959). As the size and area (0.04 mm^2) of the inducer Hensen's node graft is the same, from the average cell diameter of the host ectoblast we estimated that the graft would contact approximately 750 cells from the area pellucida and only 250 cells in the area opaca. Thus, fewer area opaca cells are in direct contact with the inducer and even fewer in case of margin of overgrowth.

The margin of overgrowth ectoblast proliferate actively (Fig. 5 E,F; Table 3) confirming earlier work (New, 1959; Downie, 1976). We now find that the proportion of ^{3}H-TdR labelled cells and mitoses decrease progressively towards the primitive streak (Fig.5, Table 3). ^{3}H-TdR labelling patterns in whole mounts and tissue sections are similar (Table 3). It is known that (Modak *et al.,* 1973) even in a uniformly tritium-labelled cell population the actual detection of labelled cells is directly dependent on cell morphology and the section thickness. We have, therefore, applied the correction formula (Modak et al, 1973) to exclude such artefacts, and find that ^{3}H-TdR labelling frequency of ectoblast is indeed lowest in the area pellucida and increases gradually to the highest ($P<0.01$) at the margin of overgrowth. Our data therefore agrees with Bellairs (1954, 1955, 1957), and do not support the suggestion (Spratt, 1966; Stern, 1979) that the primitive streak is the centre of growth. Recent data from our laboratory (Sonawane, 2000) show that the ectoblast of stages 1 to 4 of chick embryo exhibit long cell generation time (T_G = 18 h) in the area pellucida and shorter (T_G = 5-6 h) in area opaca. Thus, the time available for response to the inductive signal and its stabilisation is longer in ectoblast for area pellucida than the area opaca. It is known (Gallera, 1965, 1970, 1971; Leikola, 1976; Deshmukh *et al.,* 1998) that the exogenous HN graft induces nervous system after a minimum contact period of 4 hours with the host area pellucida but requires at least 8 hours in the area opaca. We suggest that area pellucida ectoblast, with longer cell cycle time, receive the inducer stimulus and respond better than those in area opaca and margin of overgrowth. Thus, the cell proliferative activity of the competent ectoblast (Table 3) appears to be an important parameter in determining the quantity of inductive response. This is also consistent with the observations (Fig. 2; Table 2) that the quality of induction shifts from deuterencephalic to medullary type as the grafting site is moved away from the area pellucida. Recently, we (Deshmukh and Modak, Unpublished) have found that, after a contact limited for 4-6 hours between inducing HN and the chick area pellucida ectoblast the cell cycle is activated. In any case, the proposed causal relationship between cell proliferation and state of competence needs to be further confirmed possibly by studying the effect of inhibitors and mitogens on the site specific cell proliferative activity and neural competence. In the present experiments, we have assessed the quality of induction based on morphology to those described by Sherbat (1963). Further it would be necessary to define which molecular markers distinguish increasing levels of neural induction beginning neuroidal to pallisade to medullary type.

TABLE 2

QUALITATIVE AND QUANTITATIVE ANALYSIS OF THE GRAFT-DERIVED TISSUE IN THE CHICK EMBRYO

Graft position	Number of embryos	Graft derived Neural Tissue Type *	Graft-derived tissue					Cell Population density
			Neural tube		notochord		Tissue volume ($X10^{-2}$ mm^3)	[Cell number / tissue volume]
		S D A M P	Cell no. x 10^4	Per cent mitosis	Cell no. x 10^4	Per cent mitosis		
Area Pellucida (A)	5	5 - - - -	0.91 ± 0.08	5.66 ± 2.42	0.09 ± 0.04	3.63 ± 0.39	4.07 ± 2.73	2.62 ± 0.91
Area Opaca (B)	5	4 - 1 - -	0.39 ± 0.18	4.44 ± 0.65	0.08 ± 0.03	3.44 ± 0.53	2.12 ± 0.46	2.40 ± 0.45
Margin of Overgrowth (C)	4	2 - 1 - 1	0.21 ± 0.13	3.44 ± 0.53	0.03 ± 0.03	3.21 ± 0.63	1.36 ± 0.60	2.60 ± 1.04

* Graft derived neural tissue: S, Spinocaudal; D, Deuterencephalic; A, Archencephalic; M, Medullary; P, Palisade.

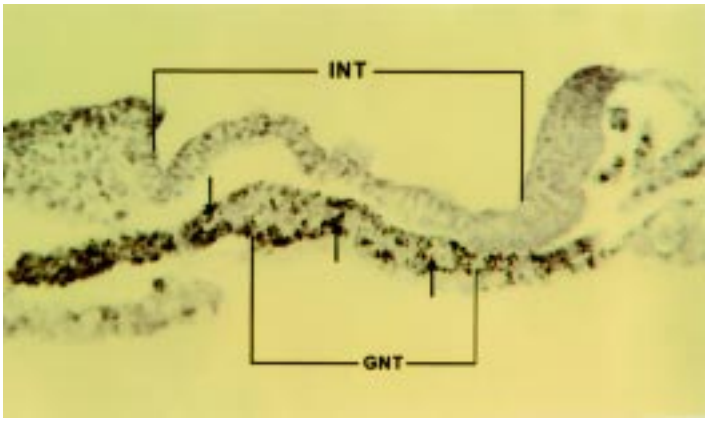

Fig. 4. Radioautograph of a transverse section of a chick embryo transplanted with ^{3}H-TdR labelled HN graft. *Note that silver grains are restricted to graft derived tissue only. The silver grains are seen in the graft-derived notochord, cut longitudinal to the host axis. INT, induced neural tube; GNT, Graft-derived notochord; arrows, indicate the silver grains.*

At the full primitive streak stage, the anterior region of the area pellucida contains the thickened cell layer or presumptive neural plate (Nicolet, 1971; Gallera, 1971; Pera and Kessel, 1997). Gallera (1971) suggested that the initial flow of the planar inductor stimulus, emanating from the Hensen's node, would gradually decrease away from the area pellucida. Morphogens, activin (Green and Smith, 1990; Blumberg and De Robertis, 1991; Green and Smith, 1992; Gurdon *et al.*, 1994) and DLX5, a homeobox gene, (Pera *et al.*, 1999), are present in form of a gradient with the highest concentration at the Hensen's node. Furthermore, RA inhibits cell population growth (Modak *et al.*, 1993) and it is at highest concentration at the HN (Hogan *et al.*, 1992). Thus the concentration gradient of morphogens appears to affect the cell proliferative activity and the differential response to an inducer. We recall the remarkable findings by polarising microscopy (Gallera and Baud, 1954) that the amphibian ectoblast exhibits sub-cellular channels in an orientation, which changes with the loss of neural competence but while treatment with a mild alkalinising agent, urea retains both, original channel orientation and neural competence. Another mild alkalinising agent, ammonium chloride induces *Xenopus* animal cap cells to express N-CAM, *otx2* and *noggin* (Uzmann, et al, 1998). Furthermore, the hepatocyte growth factor or scatter factor (HGF/SF), expressed in the Hensen's node appears to prolong the duration of the neural competence (Streit *et al.*1997).

TABLE 3
PATTERN OF DNA SYNTHESIS AND MITOSIS IN STAGE 4 CHICK ECTOBLAST

Ectoblast position on chick blastoderm	% ^{3}H-TdR labelled cells in the ectoblast			% mitosis in whole mount ectoblast
	Whole mount	Serial sections		
		observed	estimated*	
A (area pellucida border)	1.2±0.4	9.4± 1.8	16.5±4.3	2.90±0.32
B (mid area opaca)	17.2±1.2	24.7± 2.6	39.2±4.1	4.36±0.24
C (margin of overgrowth)	29.1±4.5	36.7±3.4	59.0±5.3	5.60±0.36

* % labelled nuclei in the ^{3}H-autoradiographic range estimated according to Modak *et al.,* 1973.

Based on the activation by mitogen of protein kinase C and its correlation with neuralization (Nishizuka, 1986; Davids, 1988, Otte *et al.*, 1988), Yamada (1995) proposed that ectodermal neuralization is based on the positive mitogenic signal. Among known mitogens those causing neuralizing effect is bFGF (Kengaku & Okamoto, 1993; Tiedemann *et al.*, 1994). Endogeneous secreted protein noggin is a neural inducing factor (Lamb *et al.*, 1993) and expressed in the extended state of neural competence by mild alkalinization (Uzmann *et al.*, 1998), we do not know whether noggin also acts as a mitogen. Cell surface receptors Guanine nucleotide-binding protein (Pituello *et al.*, 1991) or Follistatin (Hemmati-Brivanlou *et al.*, 1994) operate through a default pathway triggering protein kinase C (Otte *et al.*, 1988, 1989, 1991; Otte and Moon, 1992; Uzman *et al.*, 1998) and cyclic AMP (Otte *et al.*, 1989). It is also notable that intracellular alkalinization leads to the activation of protein kinase C and the cell cycle (Rozengurt, 1992). Thus changes in the cell surface and intracellular environment are

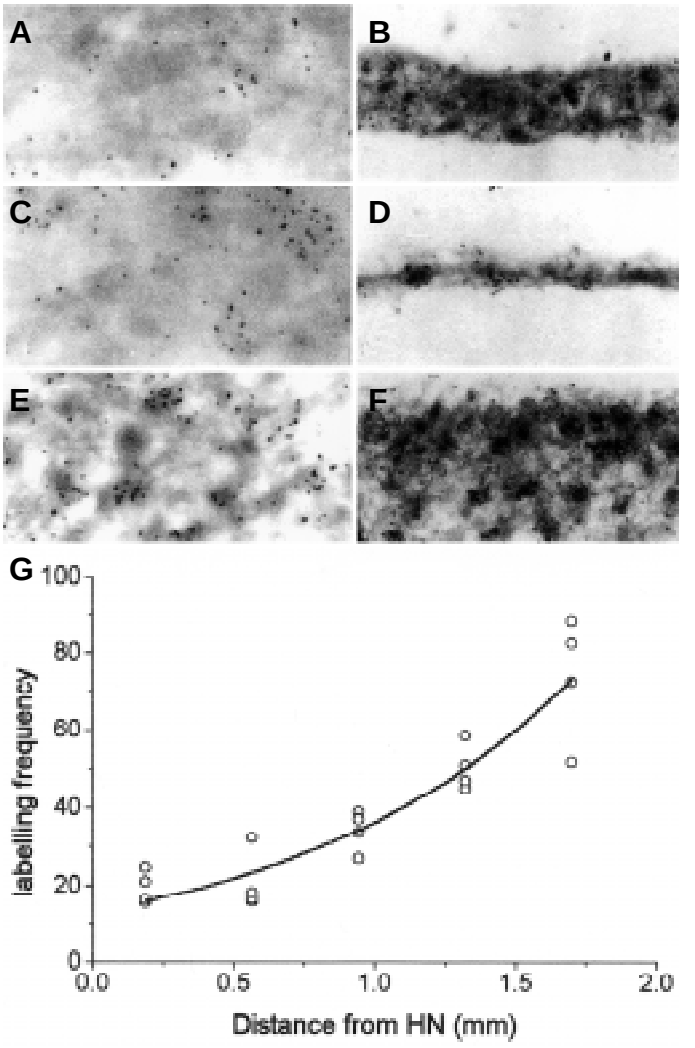

Fig. 5. Radioautographs of whole mount (A,C,E) and transverse sections (B,D,F) of chick embryo ectoblast labelled with ^{3}H-TdR in vitro. (A,B) *Position A,* **(C,D)** *position B and* **(E,F)** *position C. The graph* **(G)** *shows that the labelling frequency increases exponentially away from HN.*

relevant to the competent state. From these considerations, along with the position-specific differences in the duration of the inductor-ectoblast contact time (Gallera, 1965, 1970, 1971; Leikola, 1976; Deshmukh *et al.*, Unpublished, 2001), we suggest that the neural competence is a preparatory state for neural induction and dependent on the quantity of stimulus received through the planar flow of signals emanating from the amphibian dorsal lip or avian Hensen's node. Such an early signal may lengthen the cell cycle that is activated only by a longer exposure to the secondary neuralizing stimulus.

Materials and Methods

Full primitive streak stage 4 (Hamburger and Hamilton, 1951) blastoderms from Fresh fertilised White Leghorn chicken eggs were cultured *in vitro* (Gallera and Castro-Correia, 1960). A square (0.2mmx0.2mm) piece of the Hensen's node from stage 4 embryo was excised and placed in the area pellucida, the area opaca or the margin of overgrowth of stage 4 host blastoderm (Fig. 1). Control and grafted embryo-cultures were maintained for 20 h, fixed overnight in Bouin's and stained as whole mounts, or processed for histology. Serial sections (6 μ) were stained with Harris Haematoxylin or Mayer's hemalum. From five stage 4 blastoderms entire endo-mesoderm was scraped off and these were then fixed in acetic acid-methanol (1:3) and stained with Feulgen.

Stage 4 embryo cultures were treated for 30min with ^{3}H-TdR (4 μCi/ml; specific activity: 12.0 Ci/mmole, Brit, India), fixed in Carnoy (6 chloroform: 3 ethanol: 1 acetic acid), dehydrated and either embedded in paraffin for sectioning or whole-mounted after scraping off the endo-mesoderm. In another experiment the stage 3+ embryo were labelled with ^{3}H-TdR for 4 h (becomes stage 4 then) and the labelled HNs were grafted individually on unlabelled hosts, grown for 20 h and fixed in Carnoy.

Autoradiography: Paraffin embedded embryos were serially sectioned (6 μ) Slides were de-paraffinized, coated with Liquid emulsion NTB2 (Kodak), dried, stored in dark boxes at –20°C, and, after exposure for 4 days. developed in D-19 as before (Modak et al, 1968).

Morphometry

The cell morphology, tissue volume and cell numbers were determined as parameters of the quality and quantity of the neural induction. Outlines of induced neural tissue and graft tissue from each section were drawn to scale by Camera Lucida and the area (mm^2) was determined after multiplying by the magnification factor (0.0131). Tissue volumes in each section were estimated as the Tissue Area (mm^2) x Section thickness (6 μ).

From induced and graft-derived neural tissue in every serial section of each embryo, total number of interphase and the mitotic nuclei were counted. Similarly, diameters of 100 interphase and 25 mitotic nuclei were measured using a micrometer fitted to the eyepiece. Assuming all cells are mononucleate, cell numbers for the induced and graft-derived tissues were corrected (Aberchrombie, 1946) to avoid repetitive counting in adjacent sections and overestimation of cells. Corrected cell numbers from each section were added to obtain the total cell population size in the induced neural tissue as well as graft-derived mesoderm. Data were tested for significance by Student's "t" test (Snedecor and Cochran, 1967).

Autoradiographs were stained with Harris haematoxylin and mounted in DPX. With a background grain count of 0.4 grains per nucleus, nuclei with 3 or more grains were considered as labelled to estimate the per cent labelling frequency. Nuclear diameters were determined from 25 nuclei in each section at positions A, B and C and found to be 4.09 μ, 4.93 μ and 4.4 μ, respectively. Knowing the section thickness (6 μ), the proportion of nuclei falling in the tritium-autoradiographic range (1 μ) in sections were estimated using the formula P = [N x W'] ÷ [2R+W] (Modak *et al.*, 1973), where 2R is the nuclear diameter, W is the section thickness, W' is the ^{3}H-autoradiographic range, and N is the number of nuclei in the section and P is the proportion of nuclei falling within the tritium autoradiographic range.

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