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Timing and cell interactions underlying neural induction in the chick embryo

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SUMMARY

Previous studies on neural induction have identified regionally localized inducing activities, signaling molecules, potential competence factors and various other features of this important, early differentiation event. In this paper, we have developed an improved model system for analyzing neural induction and patterning using transverse blastoderm isolates obtained from gastrulating chick embryos. We use this model to establish the timing of neural specification and the spatial distribution of perinodal cells having organizer activity. We show that a tissue that acts either as an organizer or as an inducer of an organizer is spatially co-localized with the prospective neuroectoderm immediately rostral to the primitive streak in the early gastrula. As the primitive streak elongates, this tissue with organizing activity and the prospective neuroectoderm rostral to the streak separate. Furthermore, we show that up to and through the mid-primitive streak stage (i.e., stage 3c/3+), the prospective neuroectoderm cannot self-differentiate (i.e., express neural markers and acquire neural plate morphology) in isolation from tissue with organizer activity. Signals from the organizer and from other more caudal regions of the primitive streak act on the rostral prospective neuroectoderm and the latter gains potency (i.e., is specified) by the fully elongated primitive streak stage (i.e., stage 3d). Transverse blastoderm isolates containing non-specified, prospective neuroectoderm provide an improved model system for analyzing early signaling events involved in neuraxis initiation and patterning.

Key words: Blastoderm, Epiblast, Gastrulation, Hensen's node, In situ hybridization, Neural plate, Neural tube, Neurulation, Organizer, Primitive streak, Quail/chick chimera

INTRODUCTION

In vertebrate embryos, there seems to be a spatially, temporally and molecularly complicated set of interactions that underlies the formation of the patterned, laminar neuroepithelium. These interactions have been studied in numerous experiments aimed at either removing the neural inducer or establishing a new responding tissue by grafting potential inducers to an ectopic site. Despite recent advances in understanding the control of neural specification, the timing of neural specification remains unknown, especially in higher vertebrates. The timing has been difficult to establish because the inducer tissue and the prospective neuroectoderm are intimately associated spatially at early stages, and molecular markers for early neuroectoderm have become available only recently. We report here the timing of neural specification in the chick embryo. This timing has been discerned by using a rigorously subdivided early stage series, accurate prospective fate maps, neural markers that are expressed at early stages and a model system consisting of transverse blastoderm isolates. Our results not only reveal the timing of neural specification but also the spatial distribution of perinodal cells with organizer activity.

A pervasive dogma holds for all vertebrates, that a localized and specialized region of the embryo is established during gastrulation as an organizer (Spemann and Mangold, 1924; Waddington, 1933; Ho, 1992; Beddington, 1994; recent reviews by Lemaire and Kodjabachian 1996; Gould and Grainger, 1997; Grunz, 1997; Harland and Gerhart, 1997; Schier and Talbot, 1998; Smith and Schoenwolf, 1998). This organizer contributes cells to a number of rudiments, including the foregut endoderm, head mesoderm, notochord and floor plate of the neural tube, and it is capable of at least three main functions: inducing the neuroectoderm (neural induction), dorsalizing the mesoderm and generating the convergent extension movements that drive elongation of the neuraxis. The organizer in higher vertebrates is called the node (Hensen's node in chick) and it is found at the rostral end of the primitive streak. Cells within the rostral end of the primitive streak at early gastrula stages form the mesendoderm of the head and later, when the definitive Hensen's node has formed, they contribute principally to the midline mesodermal notochord (Selleck and Stern, 1991; Schoenwolf et al., 1992; Garcia-Martinez and Schoenwolf, 1993; Garcia-Martinez et al., 1993). The epiblast surrounding Hensen's node that does not ingress receives signals from the organizer to become neuroectoderm, whereas the epiblast beyond the reach of these signals becomes epidermal ectoderm

(Streit et al., 1995; Streit and Stern, 1999). Experiments in amphibians have demonstrated that the ectoderm expresses BMP-4, which causes the epiblast to differentiate as epidermal ectoderm. The organizer secretes several factors (e.g., follistatin, chordin and noggin) that act to inhibit the action of BMP-4, allowing the epiblast within range of the secreted factor to differentiate as neuroectoderm (reviewed by Sasai and De Robertis, 1997; Wilson and Hemmati-Brivanlou, 1997). This simple mechanism of neuroectodermal specification has not yet received strong experimental support in higher vertebrates (Mutzak et al., 1995; Winnier et al., 1995; Streit et al., 1998; Streit and Stern, 1999) and additional complexity is certain.

Numerous experiments have demonstrated that Hensen's node is sufficient to induce formation of the neuraxis. Experiments in which Hensen's node is grafted to extraembryonic regions (i.e., either to the germ cell crescent or to the inner margin of the rostral area opaca) have demonstrated that, at the late gastrula stage, Hensen's node is sufficient to cause the epiblast to form neuroectoderm and to initiate the morphogenetic movements underlying normal neurulation (e.g., Gallera, 1971; Dias and Schoenwolf, 1990; Storey et al., 1992, 1995). Other regions of the blastoderm are insufficient or less efficient at this type of induction (Waddington, 1952; Gallera, 1971). Collectively, these results suggest that Hensen's node is an organizer of the avian embryo and is responsible for induction of the neuraxis in the normal embryo. However, the insufficiency of other grafts in this assay does not necessarily mean that they are not involved in the establishment of the neuraxis in the normal embryo. The competence of extraembryonic tissue to respond to the full range of signals available to the prospective neuroectoderm has not been established, and the tissues deemed less efficient in this model system may be fully active and involved in neuroectoderm induction in the normal embryo.

To determine whether the organizer is necessary for complete neuraxis formation, many laboratories have attempted to separate the activity of the organizer from that of the rest of the blastoderm, either genetically (fish and mouse) or surgically (chick). Evaluations of knockouts of genes expressed in the mouse node (Ang and Rossant, 1994; Shawlot and Behringer, 1995) and mutants apparently lacking node and notochord in fish (Halpern et al., 1993, 1995) have demonstrated that embryos without an obvious organizer or notochord can still manage to form a respectable neuraxis, suggesting that other signaling regions are present in the gastrula that need better characterization. Node extirpation experiments in chick fail to establish what happens to prospective neuroectoderm in the absence of organizer signaling, because an organizer is reconstituted in the blastoderm after node extirpation (Yuan et al., 1995a,b; Psychoyos and Stern, 1996; Yuan and Schoenwolf, 1998). In addition, there has been accumulating evidence that signals from a separate head organizing center – presumably from the anterior visceral endoderm – are both necessary and sufficient for head organization (reviewed by Bouwmeester and Leyns, 1997; Beddington and Robertson, 1998). Attempting to unravel the complex web of signaling involved in both the formation of the organizer and in neural induction in higher vertebrates is, consequently, an area of important inquiry. Establishing a simple, new model system to assay organizer activity that uses tissue normally fated to form neuroectoderm prior to its neural specification would be desirable. However, the precise stage at which neural specification occurs in avian embryos, that is, the stage at which the prospective neuroectoderm becomes potent to form neuroectoderm, has previously not been evaluated using neural markers. Storey and co-workers (1992) set this time as prior to stage 4 (Hamburger and Hamilton, 1951). Obtaining precise knowledge of this timing, along with existing knowledge of the prospective fates of cells in the early epiblast (Schoenwolf et al., 1992; Bortier and Vakaet, 1992; Garcia-Martinez and Schoenwolf, 1993; Garcia-Martinez et al., 1993; Hatada and Stern, 1994; Callebaut et al., 1996), would provide an opportunity to isolate tissue that normally forms the neuroectoderm while it is still naive. This tissue could then be used to investigate directly the organizer activity of regions of the early blastoderm, including their ability to induce neural differentiation (assessed based on the expression of marker genes and appropriate morphology) and other changes associated with neural induction (e.g., appropriate morphogenetic movements).

We have used microsurgical manipulation of the avian gastrula to begin to investigate processes of neural induction. Based on the model used to study organizer reconstitution (Yuan et al., 1995b; S. P. Yuan and G. C. S., unpublished data), which demonstrates that caudomedial areas of the primitive streak and epiblast are required to induce an ectopic organizer, we isolated the region rostral to Hensen's node to prevent the organizer from reconstituting, while still separating the majority of the prospective neuroectoderm from Hensen's node. Once a developmental stage and region were identified that contained tissue normally fated to form neuroectoderm but incapable of doing so in isolation (i.e., naive prospective neuroectoderm), these tissue isolates were used as an assay to start investigating the tissue interactions involved in the differentiation of the rostral epiblast during avian gastrulation and early neurulation.

MATERIALS AND METHODS

Embryo culture and staging

Fertile chicken eggs were maintained at 38°C for 9-16 hours in forced-draft, humidified incubators. Embryos collected for transections (Fig. 1, experiments 1 and 2; see below) were cultured ventral-side up, off their vitelline membranes as described by Spratt (1947), with most of the caudal area opaca removed to facilitate extension of the neuraxis. Those embryos that served as recipients for grafting to the rostral extraembryonic region (Fig. 1, experiment 3; see below) were cultured intact, on their vitelline membranes according to New (1955). Embryos were staged according to the criteria of Hamburger and Hamilton (HH; 1951), with HH stage 3 (gastrula) refined according to Schoenwolf and co-workers (1992): stage 2, broad and short triangular streak (Fig. 2A); stage 3a, short and broad linear streak; stage 3b, longer and narrower linear streak; stage 3c, elongated and grooved streak (longer than the stage 3b streak, extending to the center of the area pellucida; the stage 3c streak looks like that illustrated by Hamburger and Hamilton, 1951, "stage 3+"; Fig. 2B); stage 3d, fully elongated and grooved streak (longer than the stage 3c streak, extending beyond the center of the area). Our stage 4 and subsequent stages coincide with the criteria of Hamburger and Hamilton (1951). At all stages, embryos that fell on the border between stages were grouped with the older stage.

Embryos were cultured after microsurgery on agar/albumen plates (Darnell and Schoenwolf, 1996) for approximately 24 hours. Based on the segregation of the data that we obtained, results from stages 3a to 3b were grouped as stage 3a/b; additionally, embryos at stage 4 and older were grouped as stage 4/5.

Transections

Embryos were transected on the culture dish using a cactus-needle or glass-needle knife. For experiment 1, three types of transections were done (Fig. 1). Because some tissue fated to become neuroectoderm lies lateral to Hensen's node (i.e., caudolateral to the rostral tip of the primitive streak), type A transections were made 125 μm caudal to the rostral end of the primitive streak and perpendicularly to the streak axis. Within the midline, cuts were made to exclude the primitive streak and Hensen's node from the rostral fragment. Therefore, rostral fragments of type A transections included all of the tissue fated to become neuroectoderm (V. Garcia-Martinez et al., unpublished data), whereas the entire primitive streak including the presumptive organizer and the majority of the ingressing/ingressed mesendoderm was contained in the caudal fragment. Type B transections were made just rostral to the tip of the primitive streak, thus excluding presumptive neuroectoderm caudolateral to Hensen's node. Type C transections were made similarly to type B transections, but were placed 125 μm rostral to the primitive streak. Embryos were assigned "blindly" at the time of the transection to future labeling regimens, which used one of several markers. Caudal fragments containing the organizer served as controls. After processing, data were excluded for an unlabeled rostral fragment for which the control (caudal) fragment failed to label or was lost during processing, or where notochord was detected in the rostral fragment for transections of stage 3a/b, 3c and 3d embryos (5 of 90 embryos [<6%] evaluated for the presence of notochord had this type of transection error). After exclusions, a total of 108 type A, 98 type B and 35 type C transected embryos were evaluated (Table 1; only data from embryos labeled with neural markers are listed in Table 1).

Grafting experiments

Fertile Japanese quail eggs (*Coturnix coturnix japonica)* were incubated at 38°C until embryos reached stages 3a-5. Quail embryos served as graft donors, because quail embryonic cells can be distinguished from chick cells in host embryos by immunocytochemical labeling with an anti-quail antibody (QCPN; Inagaki et al., 1993). From donor embryos, 250 μm^2 regions of the primitive streak were excised for grafting. Grafts were identified as "node" (rostral 250 μm of the primitive streak regardless of stage) or "mid-streak" (middle 250 μm of the primitive streak regardless of stage, approximately 500-750 μm from the rostral end of the streak for stage 3c embryos). Grafts were placed either under the prospective neuroectoderm of stage 3c rostral isolates (Fig. 1, experiment 2; type A and B isolates) or "under" the ectoderm in the rostral extraembryonic region of intact chick hosts (Fig. 1, experiment 3). Caudal fragments containing the host node (experiment 2) or host embryos themselves (experiment 3) served as controls.

In situ hybridization and immunocytochemistry

In situ hybridization (ISH) was carried out as previously described by Nieto and co-workers (1996), except that proteinase K, hydrogen peroxide, RNase A and the associated washes and postfixing were omitted. Immunocytochemistry (ICC) was carried out as previously described by Patel and co-workers (1989), except that the DAB reaction was sometimes altered by adding 0.025% aqueous $CoCl_2$ and 0.02% aqueous $Ni(NH_4)_2SO_4$, and hydrogen peroxide was diluted to 0.03%. Markers used consisted of the pan-neural markers Sox-2, Sox-3 and L5; rostral regional markers cNot-1 (also a Hensen's node/notochord marker), fgf-8, Frzb-1 and Otx-2; epidermal markers AP-2 and Bmp-4; the pan-streak marker brachyury; and the node and midline marker Sonic hedgehog (Shh). Sources for cDNAs and references are as follows: AP-2 (Shen et al., 1997), Bmp-4 (B. Houston), brachyury (R. Runyan), cNot-1 (P. Gruss; Stein and Kessel, 1995), fgf-8 (Crossley et al., 1996), Frzb-1 (P. Francis-West, unpublished data), Otx-2 (Bally-Cuif et al., 1995), Shh (C. Tabin; Roberts et al., 1995) and Sox-2 and Sox-3 (R. Lovell-Badge; Kamachi et al., 1995; Uwanogho et al., 1995; Rex et al., 1997). Hybridomas secreting Not-1 (Yamada et al., 1991) and QCPN (B. and J. Carlson; Inagaki et al., 1993) were obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore, MD, and the Department of Biology, University of Iowa, Iowa City, IA, under contract no. 1-HD-6-2915 from the NICHD. Supernatant containing the L5 antibody was obtained from C. Stern and M. Schackner (Streit et al., 1990, 1996).

Histology

After whole-mount ISH and/or ICC and photographic documentation, selected embryos were dehydrated, embedded in Paraplast X-tra paraffin and sectioned at 15 μm (Darnell and Schoenwolf, 1996). Sections were examined for tissue-specific morphology and expression of markers.

RESULTS

Neural specification occurs just after stage 3c

To assess the stage at which prospective neuroectoderm can form independently of the organizer in culture, we separated the prospective neuroectoderm from the rostral end of the primitive streak (prospective organizer) at progressively earlier times during gastrulation (Fig. 1, experiment 1; type A and B transections). Because work by Storey and co-workers (1992) has placed the timing of neural induction at prior to stage 4, we transected blastoderms at stage 4/5 and younger (Table 1).

Embryos from transections were labeled after culture with various neural markers, including pan-neural markers L5 (Fig. 3A), Sox-2 and Sox-3, and rostral-neural markers cNot-1, fgf-8, Frzb-1 and Otx-2 (Fig. 3B). All pan- and rostral-neural markers were expressed in 100% of the rostral isolates from embryos transected at stage 4 or older (e.g., L5, Fig. 3C; Otx-2, Fig. 3D), whereas, at stage 3d, 70% of type A and 60% of type B embryos expressed neural markers in the rostral isolate. The majority of embryos transected at stage 3c failed to express neural markers (e.g., L5, Fig. 3E; Otx-2, Fig. 3F), with 43% of type A and only 9% of type B expressing. Sections of rostral

Table 1. Experiment 1. Transections at a range of stages without subsequent grafting. Number of rostral isolates that labeled with neural markers/total number of isolates

	Stages					
	2	3a/b	3c	3d	4/5	Total
Transections						
Type A	4/4(100)	21/33(63)	9/21(43)	21/30(70)	7/7(100)	95
Type B	8/9(89)	6/13(46)	2/22(9)	15/25(60)	12/12(100)	81
Type C	–	1/8(13)	–	–	–	8

The percentage of labeled isolates is listed in parentheses.

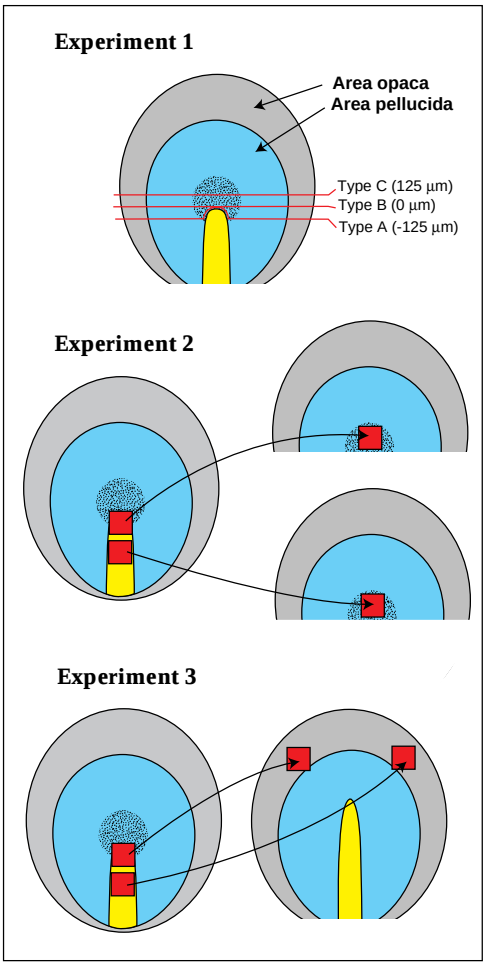

Fig. 1. Diagram of experiment 1 showing the three types of transections (type A-C). The prospective neuroectoderm is shaded. Diagram of experiment 2 showing grafts from two levels of the donor primitive streak (node and mid-streak) grafted independently to rostral isolates at stage 3c. Diagram of experiment 3 showing grafts from two levels of the donor primitive streak, and the location of grafting in the rostral extraembryonic region of the host.

isolates confirmed that, where neuroectodermal markers were expressed, tissue had the pseudostratified, columnar epithelial morphology typical of neuroectoderm (Fig. 3G, arrowheads). Conversely, where neuroectodermal markers were not expressed, tissue had a squamous morphology typical of undifferentiated epiblast (Fig. 3H, arrows).

The results from both type A and B transections revealed that the frequency of expression of neural markers progressively decreased when isolates were examined in order beginning at stage 4/5 and ending at stage 3c, with the greatest decrease being for type B transections, which lacked tissue lateral to Hensen's node. Most of the rostral isolates from stage 3c transections that did express neural markers were type A. The neuroectoderm that formed in these isolates failed to undergo the morphogenetic movements of neurulation, in contrast to embryos transected at older stages. Specifically, the neuroectoderm in stage 3c rostral isolates was limited to patches of flat neural plate at the caudal margin of the isolates (Fig. 3I), whereas isolates from embryos at stages 3d and 4 that

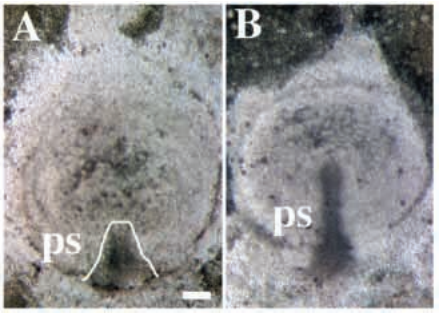

Fig. 2. (A) A stage 2 embryo showing the triangular primitive streak (outlined; ps) emerging from the caudal margin of the area pellucida. (B) A stage 3c embryo showing that the primitive streak is linear and grooved and has extended to the midpoint of the area pellucida. Bar in A for A,B, 300 μm.

contained no notochord typically formed neural vesicles, and those at stage 4 or older with notochord underwent convergent extension and projected caudally from the rostral extraembryonic ectoderm (Fig. 3J). This correlation between the presence of notochord and convergent extension was also observed when part of Hensen's node was accidentally included in the rostral fragment of transections at earlier stages (6% of cases), even when the notochord contribution was exceedingly small (Fig. 3K).

We conclude from experiment 1 on stage 3c to 4/5 embryos that at stage 3c (but not later), prospective neuroectoderm in rostral isolates is not yet potent to form neuroectoderm. Thus, specification of the neural plate occurs just after stage 3c. Type A transections at stages 3c/d displayed a higher frequency of neuroectoderm formation in the rostral isolates than did type B transections, suggesting that tissue lateral to Hensen's node may exhibit some neural-inducing/organizer activity (see below).

Prior to stage 3c, a neural inducer and prospective neuroectoderm co-exist rostral to the primitive streak in rostral isolates

To determine whether prospective neuroectoderm can be separated from tissue with neural-inducing activity at stages younger than 3c, we performed type A and B transections on blastoderms from stage 2 and 3a/b embryos (Table 1). At stage 2, transection resulted in the expression of neural markers in the rostral isolates of 100% of type A cases and 89% of type B cases (Fig. 4A, B), whereas with increasing length of the primitive streak at stage 3a/b, the frequency of expression of neural markers in the rostral isolates decreased to 63% (type A) and 46% (type B). Failure of rostral isolates to express pan-neural markers at stage 3c indicates that neural potency has not yet been established; thus at these younger stages, neural-inducing activity likely overlaps presumptive neuroectoderm and was included in the rostral isolates.

To test this possibility, we labeled type A and B stage 2 and 3a/b isolates 24 hours after culture with markers of Hensen's node, primitive streak and/or notochord (Fig. 4C-E). Such markers were expressed by 100% of stage 2 type A (3/3) rostral isolates, 87% of stage 2 type B (6/7) rostral isolates and 60% of stage 3a/b type B (3/5) rostral isolates. These numbers are in accordance with the percentage of embryos expressing

neural markers for these groups (100%, 89% and 46%, respectively; Table 1) indicating that neural-inducing (i.e., organizer) activity was indeed present in rostral isolates prior to stage 3c. By stage 3c, however, only 13% of rostral isolates expressed organizer markers (2/15; Fig. 4F), indicating that prospective neuroectoderm and neural-inducing activity could be readily separated by type A and B transections at stage 3c.

The rostral extent of neural-inducing activity prior to stage 3c

To determine the rostral extent of neural-inducing activity, and to establish whether the region of overlap between the organizer and prospective neuroectoderm is separable prior to stage 3c, stage 3a/b blastoderms were transected 125 μm rostral to the primitive streak (Fig. 1, type C isolates; Table 1). Stage 3a/b rostral isolates were probed after 24 hours of culture with the pan-neural marker Sox-2. Out of eight cases, only one rostral isolate (along with its caudal isolate mate) expressed Sox-2 (13%); in the remaining seven cases, the caudal isolates expressed Sox-2, but the rostral isolates failed to do so (Fig. 5). Consistent with this result, organizer markers failed to label stage 3a/b type C rostral isolates (0/3). Similarly, organizer markers also failed to label stage 3c type C rostral isolates (0/11). Because fate mapping has placed the rostral edge of the prospective neuroectoderm at about 250 μm rostral to the primitive streak at these stages (V. Garcia-Martinez et al., unpublished data), the most rostral 125 μm of prospective neuroectoderm can be segregated from the organizer at stage 3a-c.

Epidermal markers fail to upregulate in rostral isolates that are not specified to form neuroectoderm

As stated above, rostral isolates that expressed neural markers also formed a pseudostratified, columnar epithelial morphology typical of neuroectoderm (Fig. 3G, arrowheads). In contrast, rostral fragments that failed to express neural markers exhibited a low squamous epithelial morphology, much more reminiscent of early epiblast than of neuroectoderm (Fig. 3H, arrows). To determine whether epidermal markers were upregulated in rostral isolates lacking neural potency (isolates at stages 2-3c), we labeled type C isolates after 12 or 24 hours of culture with one of two markers (AP-2 or BMP-4; *n*=13). Although weak expression of these markers was detected along the perimeter of the isolates, the isolates did not expand or upregulate expression of these markers when compared with control embryos (data not shown), suggesting that their epiblast remained largely undifferentiated in the absence of neuralization.

In summary, our results from experiment 1 on stage 2-4/5

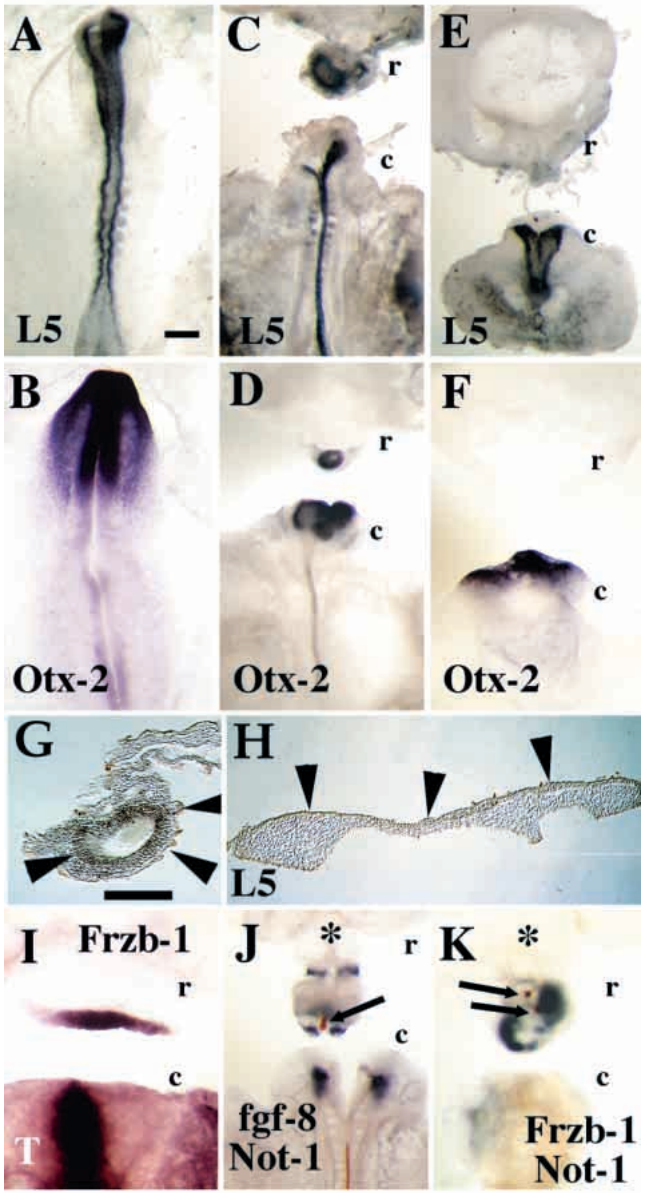

Fig. 3. Experiment 1. Control blastoderms and blastoderms transected at stages 3c-5 and labeled after culture with neural markers. (A) A control embryo at stage 9– labeled with the pan-neural marker L5 showing labeled neuroectoderm. (B) A control embryo at stage 8 labeled with the rostral neural marker Otx-2 showing labeled neuroectoderm in the head. (C) Embryo transected at stage 3d showing whole-mount L5 labeling in both rostral (r) and caudal (c) fragments (black). (D) Embryo transected at stage 3d showing whole-mount Otx-2 labeling in both rostral and caudal fragments (purple). (E) Embryo transected at stage 3c showing whole-mount L5 labeling in the caudal fragment only (black). (F) Embryo transected at stage 3c showing whole-mount Otx-2 labeling in the caudal fragment only (purple). (G) Transverse section through the rostral isolate shown in C exhibiting neuroectodermal morphology in the L5 labeled region (arrowheads). (H) Transverse section through the rostral isolate shown in E exhibiting neither neuroectodermal morphology nor L5 labeling. (I) Embryo transected at stage 3c showing expression of the neural marker Frzb-1 at the caudal margin of the rostral isolate (the caudal isolate is labeled with brachyury [T]). (J) Embryo transected at stage 5 and labeled with the neural marker fgf-8 (purple) and the notochordal marker Not-1 (brown) showing both tissues in both isolates as expected. (K) Embryo transected at stage 3a/b and labeled with the neural marker Frzb-1 (purple) and the notochordal marker Not-1 (brown; arrows) showing both tissues present in the rostral isolate (notochord is present in the rostral isolate, unexpectedly). In both J and K, the neuroectoderm has formed a midline neural structure that has projected caudally with respect to the rostral extraembryonic region (*), even though only a short piece of notochord formed in the rostral isolates (arrows). Bar in A for A-F and I-K, 300 μm; bar in G for G and H, 200 μm.

Table 2. Experiment 2. Transections at stage 3c with subsequent grafting of regions of the primitive streak. Numbers of rostral isolates that labeled with neural markers/total number of isolates

	Grafts		
	Node	Mid-streak	Total
Transections			
Type A	25/28(89)	25/26(96)	54
Type B	20/20(100)	13/13(100)	33

The percentage of labeled isolates is listed in parentheses.

blastoderms show that rostral isolates at stage 3c contain prospective neuroectoderm that is not yet specified to form neuroectoderm and that specification occurs immediately thereafter. Moreover, we show that prior to stage 3c, tissue capable of forming an organizer and, therefore, of inducing neuroectoderm, exists within 125 μm region rostral to the primitive streak, and that tissue lateral to the primitive streak also exhibits some inducing activity. It is evident from these conclusions that rostral isolates transected at 125 μm (type C) rostral to the primitive streak at stage 3a/b, and those transected at stage 3c just rostral or caudal to the node (type B and A, respectively), can serve as useful models to study induction and patterning of the prospective neuroectoderm.

Primitive-streak grafts to rostral isolates rescue the expression of neural markers

To determine whether expression of neural markers can be rescued, and to begin to determine which tissues are capable of causing such rescue, in experiment 2, we grafted two levels of the primitive streak into rostral isolates that are not yet specified to form neuroectoderm (Fig. 1). Grafting to rostral isolates differs from the traditional approaches used to investigate organizer activity (i.e., grafting to extraembryonic regions) in that the responding tissue is non-specified *prospective* neuroectoderm.

Grafts with surface areas of 250 μm^2 were taken from the rostral primitive streak (i.e., the level containing Hensen's node) and placed under the prospective neuroectoderm of stage 3c rostral isolates. Our intent was to use these grafts to determine whether the traditional organizer could rescue expression of neural markers in non-specified prospective neuroectoderm. In addition, grafts with surface areas of 250 μm^2 were taken from the mid-primitive streak and placed under the prospective neuroectoderm of stage 3c rostral isolates. Our intent was to use these grafts to determine whether a region not typically recognized as having organizer activity could rescue expression of neural markers in non-specified prospective neuroectoderm. All pan- and rostral-neural markers were expressed at high frequency in rostral isolates containing either node (89-100%) or mid-streak (96-100%) grafts from all stages tested (Fig. 6A,B; Table 2). Thus, both the traditional organizer level and other non-organizer levels of the primitive streak exhibit organizer activity in this assay.

Table 3. Experiment 3. Grafting of regions of the primitive streak to the rostral extraembryonic region of stage 3d hosts. Number of ectopic structures that labeled with neural markers/total number of grafts

Grafts		
Node	Mid-streak	Total
17/17 (100)	6/13 (46)	30

The percentage of labeled ectopic structures is listed in parentheses.

Primitive-streak grafts to rostral extraembryonic regions induce the expression of neural markers

To compare this new model system with the traditional assay, in experiment 3, we again grafted two levels of the primitive streak, but this time grafts were placed into the extraembryonic region (Fig. 1; Table 3). Hensen's node

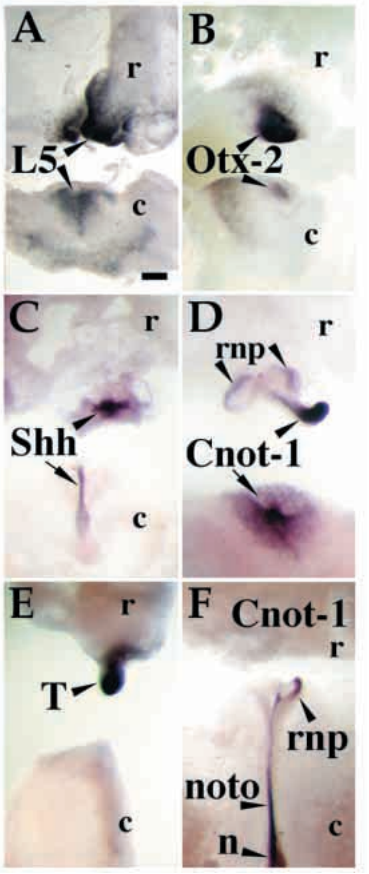

Fig. 4. Experiment 1. Stage 2-3c transections labeled with neural and other markers. (A) Embryo transected at stage 2 showing whole-mount L5 labeling in both rostral (r) and caudal (c) fragments (arrowheads). (B) Embryo transected at stage 3a/b showing whole-mount Otx-2 labeling in both rostral and caudal fragments (arrowheads). (C-E) Labeling with node/notochord/primitive streak markers, Sonic hedgehog (Shh, C), cNot-1 (D) or brachyury/T (E) after culture of embryos transected at stage 2 shows the expression of markers (arrowheads) in rostral isolates (rnp, dorsal fold of rostral neural plate). In C and D, markers also labeled the caudal isolate (arrows). Labeling of the caudal isolate in E (i.e., the expected result) was weak or absent in this particular experimental case. Labeling with the node/notochord marker cNot-1 (F) after culture of an embryo transected at stage 3c shows labeling in the caudal isolate in the node (n), notochord (noto) and dorsal lip of the rostral neural plate (rnp), whereas cNot-1 was not expressed in the rostral isolate. Bar in A for A-F, 200 μm.

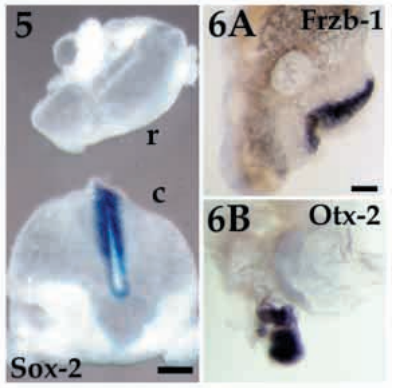

Fig. 5. Experiment 1, type C transection. Stage 3b transection labeled with the pan-neural marker, Sox-2. Sox-2 labeling was present in the caudal (c) but usually not rostral (r) isolate of stage 3a-3c embryos transected at level C. Bar, 300 μm.

Fig. 6. Experiment 2. Rostral isolates containing non-specified prospective neuroectoderm (stage 3c) shown 24 hours after grafting of Hensen's node or the mid-streak. (A) Stage 5 donor mid-streak graft resulted in the expression of the neural marker Frzb-1. (B) Stage 3d donor Hensen's node graft resulted in the expression of the neural marker Otx-2 (purple). Bar in A for A, B, 200 μm.

induced neural markers 100% of the time (*n*=17; Fig. 7A,B). Additionally, mid-streak grafts to the extraembryonic region were capable of inducing neural markers, but they did so in only 46% of the cases (*n*=13; Fig. 7C-F). Therefore, we conclude that both Hensen's node and the more caudal primitive streak are capable of de novo (i.e., initiating) neural induction, but the sensitivity of the extraembryonic region to respond to this activity is diminished compared to the rostral isolates.

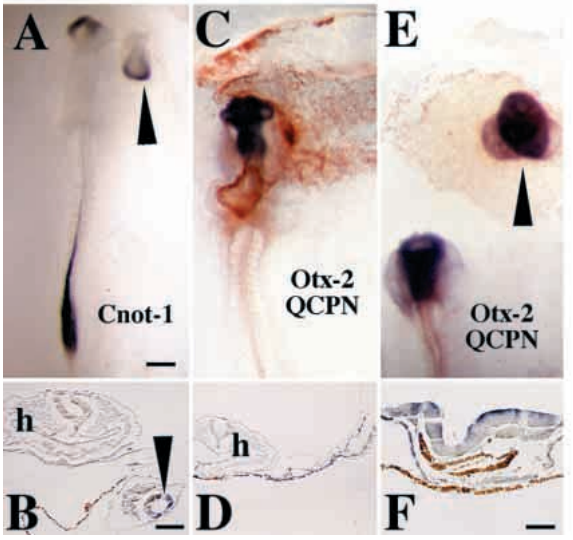

Fig. 7. Experiment 3. Grafts of mid-streak have organizer activity when grafted to the rostral extraembryonic region. (A) A Hensen's node graft from a stage 3a/b donor induced neural expression of the marker cNot-1 (arrowhead) in the rostral extraembryonic ectoderm. (B) Cross section of the same embryo shows an ectopic (induced) neural tube (arrowhead) and graft cells (brown cells in the endoderm). (C) A mid-streak graft from a stage 3a/b donor failed to induce neuroectoderm when grafted to the rostral extraembryonic region. The graft (brown) contributed to extraembryonic endoderm and mesoderm of the host heart. (D) Cross section of the embryo shown in C reveals graft cells (brown) in the endodermal layer. (E) A mid-streak graft from another stage 3a/b donor induced the neuroectodermal marker Otx-2 (purple; arrowhead) when grafted to the rostral extraembryonic region. (F) Cross section of E shows that the graft contributed to the endoderm (brown). Bar in A for A, C and E, 300 μm; bar in B for B and D, 100 μm; bar in F for F, 50 μm.

DISCUSSION

Induction of the neuroectoderm is a defining feature of organizer activity and early axis formation (e.g., Harland and Gerhart, 1997; Smith and Schoenwolf, 1998). The precise timing and mechanism of formation of neuroectoderm in amniotes, however, has been largely left unresolved. As with other types of embryonic induction, induction of the neural plate likely requires that the tissue first become competent. The establishment of neuroectodermal competence presumably occurs between HH stages 2 and 3+, because the expression of L5 (a marker of neural competence) shows increasing expression in the rostral epiblast at these stages (Streit et al., 1997). Once competence is established, a probable scenario is that interactions with adjacent tissue and/or diffusible signaling molecules lead to induction of the competent tissue, which ultimately becomes specified to form neuroectoderm. Here, we focus directly on determining the stage at which neural specification occurs, and whether neural-inducing activity can be separated from the traditionally defined organizer activity. We also show using molecular markers that the primitive streak itself is capable of inducing the formation of neuroectoderm in competent tissue. Finally, we present a new model system, transverse blastoderm isolates, for analyzing early induction and patterning of the avian embryo.

Neural induction and potency

We show using a new model system, transverse blastoderm isolates, that the area rostral to the primitive streak is potent to self-differentiate as neuroectoderm when separated from the organizer at stage 3d, but is not potent at earlier stages; thus, the neuroectoderm becomes specified between stages 3c and 3d. The tissue interactions that cause neural induction have been investigated in the present study by grafting tissue to rostral blastoderm isolates (experiment 2). The rostral primitive streak, which has been defined as the chick organizer (Waddington, 1933), is capable of inducing neuroectoderm in rostral isolates containing non-specified prospective neuroectoderm. This induction occurs vigorously even prior to the time that the rostral primitive streak becomes committed to form notochord, and at a time when this level of the primitive streak is forming predominantly endodermal cells and not notochord (V. Garcia-Martinez et al., unpublished data). Similarly, the mid-streak also has a strong ability to induce neuroectoderm, even at younger stages when the mid-streak is fated to form predominantly cardiac mesoderm and its underlying endoderm (Garcia-Martinez and Schoenwolf, 1993; V. Garcia-Martinez et al., unpublished data).

The potential role of endoderm as a neural-inducing tissue was suggested in the 1930s (Waddington and Schmidt, 1933), and this role has been supported by more recent experiments

using reliable markers to distinguish donor and host cells (Dias and Schoenwolf, 1990). In the latter experiments, the frequency of neural induction after grafting nodes of various stages was directly correlated with the quantity of endoderm derived from the graft. The type of endoderm showing this correlation was derived from the organizer and was, therefore, rostral embryonic (i.e., definitive) endoderm. Recently, the rostral extraembryonic (visceral) endoderm has also received attention for its role in head formation in other vertebrates (reviewed by Bouwmeester and Leyns, 1997; Beddington and Robertson, 1998). Because we have shown that the rostral blastoderm, which includes the rostral extraembryonic endoderm, lacks the ability to express neuroectodermal markers in embryos transected at stage 3c (experiment 1), we have demonstrated that, in chick, any signaling centers present in the rostral isolate prior to stage 3d are insufficient to independently induce neuroectoderm at this stage. This does not rule out the involvement of rostral signaling centers in neural induction/patterning, or in establishing the organizer. In fact, rostral endoderm is known to have a signaling role in the formation of the heart (Schultheiss et al., 1995), and may well be involved in other early determination and patterning events in the chick as suggested for the rostral mesendoderm of the mouse (Ang and Rossant, 1993).

The possibility that prospective cardiac mesoderm induces neuroectoderm (i.e., has organizer activity) or induces other cells to have organizer activity is a new consideration based on this work and on the fate maps of prospective cardiac mesoderm. We and others (Yuan et al., 1995a,b; Psychoyos and Stern, 1996; Yuan and Schoenwolf, 1998) have established that tissues near the node are capable of forming organizer/notochord under appropriate circumstances. Specifically, formation of an ectopic organizer is induced when the normal organizer is removed. Rostral isolates containing non-specified neuroectoderm clearly lack the ability to form an organizer in isolation. However, we have shown here that cells of the mid-streak at stages 3b to 3c, which are fated to become cardiac mesoderm and underlying endoderm (Garcia-Martinez and Schoenwolf, 1993), can induce neuroectoderm in rostral isolates. Future studies will consider whether this induction is direct (i.e., this region can act as an organizer) or indirect (i.e., this region can induce an organizer in the rostral isolate). Induction of neuroectoderm in the rostral extraembryonic region in type 3 experiments indicate the former is most likely.

We also show for the first time that, at stages 2-3c, tissue lateral to Hensen's node as well as rostral to this structure has the ability to induce neuroectoderm. Further studies are underway to characterize these cell populations and their specific role in neural induction and regional patterning.

Separating the different functional components of the organizer

Three main functions have been proposed for the organizer: inducing the neuroectoderm, generating the convergent extension movements that drive elongation of the neuraxis and dorsalizing the mesoderm (Gallera, 1971; Gerhart et al., 1991; Keller et al., 1992; Harland and Gerhart, 1997; Poznanski et al., 1997). It has not clearly been determined whether these activities can be separated as individual inductive events, or whether they are inseparable. Because the organizer changes its cellular composition over time, forming head mesendoderm early and later contributing principally to the notochord (Selleck and Stern, 1991; Schoenwolf et al., 1992; Garcia-Martinez and Schoenwolf, 1993), it is possible that induction of neuroectoderm is controlled by one organizer cell type, whereas convergent extension and dorsalization are controlled by other organizer cells. Separating organizer function has not been possible in traditional experiments in which Hensen's node is grafted to a rostral extraembryonic region, because an entire new axis is formed. In contrast, our results show that when node or mid-streak tissue which produces endoderm and not notochord (Schoenwolf et al., 1992; Garcia-Martinez and Schoenwolf, 1993), is grafted next to non-specified prospective neuroectoderm, neuroectodermal gene expression and neural plate morphology can be induced in the absence of axis formation and convergent extension. This supports the concept that induction of the neuroectoderm can be separated from other activities of the organizer. Similarly, axis extension was observed only when notochord cells were present in the rostral isolates, either from grafted older nodes or after transection errors, indicating that axis extension may be specifically attributable to this cell type. Further characterization of the induction of notochord within the node and its involvement in convergent extension will be reported elsewhere (D. K. D. and G. C. S., unpublished data).

Finally, our results do not support the possibility that the epiblast will self-differentiate epidermis in the absence of neuralization. Using transverse blastoderm isolates that have not been specified to form neuroectoderm (experiment 1), we show that, in the absence of neuralization, epidermalization is not upregulated or expanded. This result is fully consistent with the recent findings of Pera and co-workers (1999). They show that induction of epidermis is triggered by the midline and that the effect is transferred via the neural plate to the periphery. In transverse blastoderm isolates containing non-specified prospective neuroectoderm, we show that neither midline differentiation nor neural plate differentiation occurs. Consequently, as expected from the results of Pera and co-workers (1999), the epiblast remains undifferentiated.

Neural specification in transverse blastoderm isolates: neural induction or maintenance?

It is conceivable that neural induction is initiated earlier than our experiments indicate, and that what our rostral blastoderm isolates reveal is a requirement for a neural maintenance factor from the organizer or more caudal primitive streak. However, our results using grafts of primitive streak to rostral extraembryonic regions (experiment 3), as well as the previous results of many others (e.g., Gallera, 1971; Dias and Schoenwolf, 1990; Storey et al., 1992, 1995), clearly show that Hensen's node (and mid-streak as shown here) can initiate neural induction. Thus, it is reasonable to suggest that Hensen's node (and mid-streak) can also initiate neural induction in rostral blastoderm isolates containing non-specified prospective neuroectoderm. Moreover, the fact that rostral blastoderm isolates lacking organizer tissue are specified to form neuroectoderm by stage 3d, and can do so in isolation from the very tissues that can initiate neural induction when transplanted to the rostral extraembryonic region, argues that a long-term maintenance signal is not required for neural induction. However, it cannot be ruled out that a maintenance factor, located rostral to the organizer at stage 3d but previously

co-existing with it, is responsible for the potency of 3d rostral isolates in our experiments. Furthermore, we used several so-called “neural” markers in this study (as well as neural plate morphology), but it is unclear to what extent these markers represent competence factors, neural differentiation factors or genes that are expressed as a result (rather than the cause) of neural differentiation. This issue will be important to resolve in future studies because some of these markers are expressed in avian embryos prior to stage 3d, the stage by which our result demonstrate that neuroectoderm is specified. For example, Sox-3 is first expressed at stage 1 (i.e., prior to formation of the primitive streak), whereas Sox-2 is not expressed until stage 4 (the fully elongated primitive streak stage; Rex et al., 1997). By contrast, Otx-2 is first expressed in epiblast at stage 3+ (equivalent to stage 3c; Bally-Cuif et al., 1995), the stage at which our results suggest epiblast is becoming specified. Considerable work remains to elucidate the particular interactions and responses involved in neural specification in higher vertebrates; our new model system provides unique opportunities to unravel these mysteries.

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