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Axial mesendoderm refines rostrocaudal pattern in the chick nervous system

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SUMMARY

There has long been controversy concerning the role of the axial mesoderm in the induction and rostrocaudal patterning of the vertebrate nervous system. Here we investigate the neural inducing and regionalising properties of defined rostrocaudal regions of head process/prospective notochord in the chick embryo by juxtaposing these tissues with extraembryonic epiblast or neural plate explants. We localise neural inducing signals to the emerging head process and using a large panel of region-specific neural markers, show that different rostrocaudal levels of the head process derived from headfold stage embryos can induce discrete regions of the central nervous system. However, we also find that rostral and caudal head process do not induce expression of any of these molecular markers in explants of the neural plate. During normal development the head process emerges beneath previously induced neural plate, which we show has already acquired some rostrocaudal character. Our findings therefore indicate that discrete regions of axial mesendoderm at headfold stages are not normally responsible for the establishment of rostrocaudal pattern in the neural plate. Strikingly however, we do find that caudal head process inhibits expression of rostral genes in neural plate explants. These findings indicate that despite the ability to induce specific rostrocaudal regions of the CNS de novo, signals provided by the discrete regions of axial mesendoderm do not appear to establish regional differences, but rather refine the rostrocaudal character of overlying neuroepithelium.

Key words: Neural induction, Rostrocaudal pattern, Chick embryo, Head process, Axial mesendoderm, Notochord

INTRODUCTION

The axial mesoderm that first emerges from the avian organiser region, Hensen's node, consists of two cell populations which quickly resolve into the rostrally located prechordal mesendoderm and the prospective notochord. This early emerging notochord is called the head process (Lillie, 1952; also see Spratt, 1947) and remains continuous with the prechordal mesendoderm beneath the neural plate to a rostral limit at the level of the prospective caudal diencephalon. The caudal end of the head process is defined by the position of Hensen's node at the onset of caudal regression. This movement begins at head fold stages (Hamburger and Hamilton, 1951, HH stage 6) when the node is level with the future position of the otic vesicle and the notochord proper is then laid down in the wake of the regressing node. In this way the head-process/notochord comes to underlie almost the entire length of the central nervous system (CNS) and it is the intimate contact between these tissues that has led to intensive investigation of the role of the notochord in the induction and patterning of the vertebrate nervous system (reviewed for example in Tanabe and Jessell, 1996). In particular, such studies provide conflicting evidence for the ability of different rostrocaudal levels of axial mesendoderm to induce neural tissue and to convey regional identity to overlying neuroepithelium.

Grafts of young (HH3-4) and head-fold stage (HH6) Hensen's nodes induce distinct rostrocaudal regions of the CNS, early nodes induce the full rostrocaudal extent of the neural tube (including forebrain and midbrain) while older nodes are weaker inducers and generate only caudal regions (hindbrain and spinal cord) (Kintner and Dodd, 1991; Storey et al., 1992). This suggests that tissues emerging from the node between these stages might provide signals that induce rostral CNS. It has recently been shown that while prechordal tissue can specify forebrain character, it is not a source of neural inducing signals (Dale et al., 1997; Foley et al., 1997, but see Pera and Kessel, 1997). Hara (1961, 1978) suggests that the head process retains the neural inducing activity lost by the older node. However, as Hara's grafts of different stage head process were juxtaposed with embryonic epiblast, which may have already been exposed to neural inducing signals, these experiments are not a true test of neural inducing activity. These experiments were also carried out prior to the availability of neural- or region-specific molecular markers and therefore the precise inducing and regionalising properties of these tissues have yet to be defined.

As the head process extends beneath the neural plate it may

also play a role in defining the rostrocaudal character of this neuroepithelium. The signalling properties of different rostrocaudal levels of axial mesendoderm were first assessed in the amphibian embryo by Mangold (1933), who demonstrated that different regions of this tissue can induce distinct regions of the CNS. These observations have since been refined by the use of region-specific molecular markers which reveal that such inductions lead to the formation of broad regional domains that share some characteristics. For instance, both rostral and caudal notochord can induce expression of the metencephalic marker Engrailed 2 (Hemmati-Brivanlou et al., 1990). This finding is consistent with the gradual restriction of rostrocaudal character observed in the amphibian neuroepithelium (Saha and Grainger, 1993) and suggests that the axial mesendoderm does not provide precise regionalising signals. Experiments specifically designed to assess the regionalising properties of the avian notochord (Grapin-Botton et al., 1995; Fukushima et al., 1996; Matise and Lance Jones, 1996) also suggest that it does not convey rostrocaudal character in late stage (HH10-14) embryos (see also Muhr et al., 1997). Recent experiments carried out on younger embryos suggest that the early, emerging head process is required for the expression of *En1* (Shamin et al., 1999). This finding, however, is inconsistent with the node removal experiments which reveal that broad rostrocaudal subdivisions of neural plate, including En2 expression domain, can form in the absence of underlying notochord/head process (Darnell et al., 1992; Spann et al., 1994). Thus, studies in different animals and the use of different assays present contradictory accounts of the role of axial mesoderm in the induction and rostrocaudal patterning of the CNS.

Further, it has recently been shown that paraxial mesendoderm (pre- and post- overt segmentation) can caudalise already induced rostral neural tissue in a number of vertebrate embryos (Itasaki et al., 1996; Bang et al., 1997; Muhr et al., 1997; Woo and Fraser, 1997; Gould et al., 1998). Indeed, while prechordal axial mesendoderm can confer rostral character to caudal neural tissue (Dale et al., 1997, 1999; Foley et al., 1997; Pera and Kessel, 1997) it has been shown that rostral paraxial mesendoderm also provides rostralising signals (Dale et al., 1997) demonstrating that some regions of both axial and paraxial mesendoderm act to regulate rostrocaudal character.

Negative as well as positive regulation of the rostrocaudal character of neuroepithelium has also been demonstrated in grafts containing axial and paraxial mesendoderm in the mouse (Ang and Rossant, 1993; Ang et al., 1994). The idea that the notochord may be a source of such inhibitory signals has also been raised by Poznanski and Keller (1997) who describe the local inhibition of *Hoxb1* expression in the amphibian neural tube by signals provided by the underlying notochord. In the mouse embryo, the ability of posterior mesendoderm to inhibit expression of rostral genes can be mimicked by retinoic acid (Ang et al., 1994), a signalling molecule that can also inhibit expression of the rostral gene *otx-2* in the chick embryo (Bally-Cuif et al., 1995; and see Maden et al., 1996; Bang et al., 1997). Fibroblast growth factor (FGF) and Wnt3A are other signalling molecules that can suppress expression of rostral neural genes and elicit expression of caudal neural genes: in the frog, FGF (Cox and Hemmati-Brivanlou, 1995; Kolm et al., 1997), and Wnt3A (McGrew et al., 1995), and FGF in the chick (Rodriguez-Gallardo et al., 1996; Henrique et al., 1997; Storey et al., 1998). However, other studies present contradictory findings with respect to the role of FGF. Experiments in the zebrafish embryo suggest that FGF cannot mimic the caudalising effects of non-axial mesendoderm (Woo and Fraser, 1997) and in the chick embryo there is also evidence that FGF signalling acts indirectly to induce caudal neural markers (Muhr et al., 1997; Storey et al., 1998; also see Pownall et al. 1996). Thus, although we have a number of candidate signalling molecules that may induce and/or impose rostrocaudal pattern on the CNS it is still unclear which tissues provide these signals and at what time they act during development.

We have characterised the neural inducing and regionalising activities of defined mesendodermal cell populations and also assessed the ability of overlying neural plate to respond to such signalling during normal development. We show that neural-inducing signals are present in axial mesendoderm and that different rostrocaudal levels of this tissue induce distinct regions of the CNS in competent epiblast. While rostral head process induces only the midbrain/hindbrain boundary (metencephalic) region of the CNS, caudal head process induces neural tissue of caudal hindbrain and rostral spinal cord character. However, rostral and caudal head process do not induce any of the region-specific genes assessed in explants of the overlying neural plate. This suggests that signals from the head process (at HH6-7) do not establish regional neural character during normal development. On the other hand, caudal head process was found to inhibit expression of genes found rostral to the region of the CNS under which it normally comes to lie. Our findings suggest two roles for head process tissues: the reinforcement of earlier neural inducing signals and the later provision of inhibitory signals that serve to refine rostrocaudal pattern within the neuroepithelium. This study also shows that the inhibition of rostral character and the induction of caudal neural genes are experimentally separable, opening the way to the identification of signalling molecules responsible for these different patterning events.

MATERIALS AND METHODS

Dissection and grafting procedure

Fertile hen's eggs (Warrens) were incubated at 38°C for 12 hours to give host embryos of HH3-3+ and were prepared in New culture (New, 1955), modified as described by Stern and Ireland (1981). In all cases (except where stated otherwise) grafts were derived from quail embryos which had been incubated at 38°C for 24 hours to give embryos of HH6-7 (head-fold stages). The region to be grafted as defined in Fig. 1A was dissected in calcium- and magnesium-free (CMF) Tyrode's saline containing 0.1% Trypsin (Difco 1:250) in order to separate the mesendoderm and neural ectoderm layers. All donor tissues were grafted into the area opaca in contact with the extra-embryonic epiblast, at the level of the host node (although there is a small variation in the location of grafts at the end of the incubation time, we have not observed differences in the repertoire of genes expressed by and in response to grafts). The head process tissue was grafted in such a way so that the mesoderm, and not the endoderm, was adjacent to the extra-embryonic epiblast. The rostral head process (RHP) grafts were shown to be free of prechordal tissue, molecularly defined by the gene *goosecoid* (Izpisúa-Belmonte et al. 1993) (Fig. 1B,C). In addition, presomitic tissue as defined by the gene *paraxis*

(Barnes et al., 1997) was not included in the caudal head process (CHP) grafts (Fig. 1D,E), which do however, express the notochord marker Brachyury (see Fig. 7A,B). In experiments to test the ability of head process or paraxial mesendoderm to pattern the already induced neural plate, explants were dissected separately and combined (or recombined) as required and grafted into the extra-embryonic epiblast. Neural plate explants were wrapped around the mesodermal tissues to ensure close contact between these tissues and to reduce potential induction of host extra-embryonic epiblast. All host embryos were subsequently maintained in New culture for a further 24-30 hours.

Criteria used to define neural induction and regionalisation

A combination of morphological and molecular criteria were used to define neural tissue and its regional character. Neural tissue was identified by its characteristic morphology (neural tube, neural plate or a raised columnar epithelium) and by the expression of the pan-neural marker *Sox2* (Uwanogho et al., 1995; see Streit et al., 1997). Distinct rostrocaudal regions of the CNS were identified by the expression of eight distinct region-specific molecular markers (Fig. 2).

Whole-mount in situ hybridisation

Embryos for whole-mount in situ hybridisation were fixed in phosphate-buffered 4% formaldehyde and hybridisation performed with digoxigenin- (DIG) labelled riboprobes (modified after Izpisúa-Belmonte et al., 1993). Anti-DIG alkaline phosphatase-conjugated antibody (Boehringer) was visualised with NBT and BCIP. Embryos were then washed twice in PBS and post-fixed and stored in phosphate-buffered 4% formaldehyde. All probes were found to give the same patterns of expression in chick and quail embryos.

Immunocytochemistry

Embryos were fixed for 1 hour in phosphate-buffered 4% paraformaldehyde (pH 7.0) for incubation with En-2 monoclonal antibody 4D9 (Patel et al., 1989). Immunolabelling of whole-mount embryos with antibody 4D9 followed a standard protocol (see Storey et al., 1992). Briefly, following fixation, endogenous peroxidase activity was blocked with 0.25% hydrogen peroxide in phosphate-buffered saline (PBS), pH 7.4. Embryos were rinsed in PBS, then blocked in PBT (PBS containing 0.2% bovine serum albumin, 0.5% Triton X-100, 0.01% thimerosal and 5% heat inactivated normal goat serum. Supernatant was added 1:1 and embryos incubated overnight at 4°C. After extensive washing in PBT, embryos were incubated in peroxidase- (HRP) conjugated goat anti-mouse IgG antibody (Jackson ImmunoResearch) overnight. Embryos were then washed in PBS and rinsed in 0.1 M Tris (pH 7.4) prior to reacting with 3, 3′-diaminobenzidine tetrahydrochloride (DAB; 1 mg ml in 0.1 M Tris, pH 7.4) with H_2O_2 (0.001%).

Immunolabelling with QCPN

Embryos to be labelled with the quail-specific antibody QCPN (obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Pharmacology and Molecular Sciences, John Hopkins University School of Medicine, Baltimore, MD and the department of Biological Sciences, University of Iowa, Iowa City, IA, under contract N01 HD-6-2915 from the NICHD) were fixed in phosphate-buffered 4% formaldehyde for 1 hour and then incubated in 0.25% H_2O_2 in PBS for 30 minutes. They were then washed several times in PBS, and blocked in PBT for 2 hours. QCPN antibody was added as culture supernatant diluted 1:10 in the blocking buffer and incubated overnight at 4°C. Embryos were then rinsed in PBT for 4 hours and incubated overnight in goat anti-mouse IgG-HRP (Jackson; 1:500) at 4°C. Following washes in PBT for 4 hours the embryos were then rinsed twice in 100 mM Tris-HCl (pH 7.4). Finally, the embryos were incubated for 30 minutes in DAB (200 μg/ml) and H_2O_2 added to a final dilution of 1:10,000. Staining was stopped and intensified by washing in tap water.

QCPN labelling prior to in situ hybridisation

To avoid loss of mRNA during immunocytochemistry for QCPN, all solutions, following the 1 hour H_2O_2 0.25% step (above), contained 1 M LiCl until the end of the DAB reaction (Stern, 1998). DAB was washed away with DEPC-treated sterile H_2O. In situ hybridisation was then carried out as described above.

DiI labelling

Small groups of cells were labelled with DiI (1,1′-dioctadecyl-3,3,3′, 3′ tetramethyl indocarbocyanine perchlorate; Molecular Probes), by iontophoresis (Erskine et al., 1998). Following incubation, all DiI-labelled embryos were fixed in 4% buffered formol saline, pH 7.0. DiI-labelling was revealed by epifluorescence (peak excitation 484 nm). Embryos were then either (a) stored in fixative in the dark; (b) following a 1 hour fixation, washed in 1% sucrose/PBS overnight prior to embedding in OTC and sectioned frozen or (c) washed in 100 mM Tris-HCl (pH 7.4) then incubated for 30 minutes in DAB (200 μg/ml) before being photo-oxidised and then embedded in wax prior to sectioning at 10 μm.

RESULTS

The fate of rostral and caudal head process and overlying neuroepithelium

The fates of tissues to be grafted in this study were established using the lineage tracer DiI. Small groups of (2-5) cells were labelled in the rostral and caudal head process and overlying neuroepithelium at HH6-7 (defined in Fig. 1 and see Fig. 3A-E, J-N for time 0) and their fates assessed following incubation to HH10-11. While rostral head process (RHP) gives rise to cells that lie beneath the diencephalon/anterior hindbrain (12/12 cases, Fig. 3F,H), caudal head process (CHP) comes to underlie the anterior spinal cord adjacent to somites 3-12 (10/10 cases; Fig. 3G,I). The neural plate overlying the RHP corresponds to the forebrain at the level of the optic vesicle (12/12 cases; Fig. 3O,Q) and neural plate overlying the CHP contributes to posterior hindbrain (15/15 cases; Fig. 3P,R). These results are summarised in Fig. 3A,J and show that the axial mesoderm and the neuroepithelium shift relative to each other during development, resulting in the neural plate acquiring a more rostral position with respect to the underlying head process.

Neural inducing activity varies along the length of the head process and is absent from paraxial mesoderm

The neural inducing ability of rostral and caudal head process derived from HH 6-7 quail donor embryos was assessed by grafting these tissues to an ectopic site in a stage 3-3+ chick host embryo (Fig. 4). Grafts were placed in direct contact with extraembryonic epiblast, which can respond to neural inducing signals provided by grafts of Hensen's node (Waddington, 1932; Gallera, 1970; Storey et al., 1992, 1995). Graft-derived cells were distinguished from host chick cells using the anti-quail antibody QPCN and the extent to which extraembryonic epiblast acquired neural morphology in response to grafts was assessed by scoring for the formation of: (i) a neural tube (Fig. 4A,B), (ii) a neural plate (Fig. 4C) or (iii) a raised epithelium (Fig. 4D,E; see Table 1). RHP induces extraembryonic epiblast

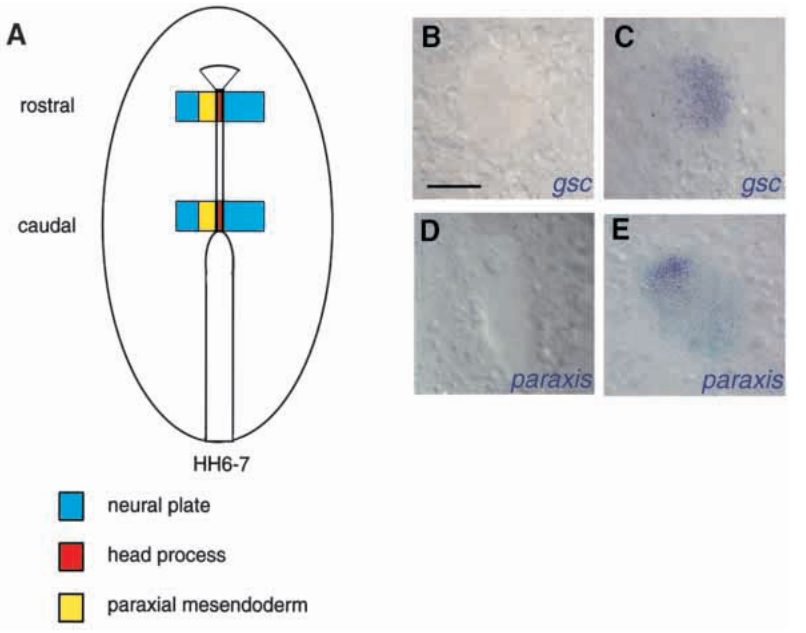

Fig. 1. Definition of grafted tissues. (A) Prospective notochord formed by axial mesoderm that has emerged and moved rostrally from the node is defined as the head process (Spratt, 1947). We have divided this tissue into four equal lengths. At HH6-7 the rostral quarter is defined as rostral head process. At this stage the caudal quarter is a mixture of both head process and the prospective 'notochord proper' (which is laid down subsequent to node regression at about HH6), but for simplicity we have called this tissue caudal head process. The axial mesoderm at this stage of development is intimately associated with the underlying endoderm layer and our head process grafts therefore consist of axial mesendoderm. Paraxial mesendoderm consisted of a block of mesendodermal tissue adjacent to either the rostral or caudal head process. Rostral and caudal neural plate explants consisted of both dorsal and ventral neuroectoderm present at the same level as the rostral or caudal head process. Rostral head process grafts were assessed for the presence of contaminating (*goosecoid*- (*gsc*) expressing) prechordal tissues. (B) RHP grafts placed in contact with the extra-embryonic epiblast for 4 hours, did not contain *gsc*-expressing cells (n=6), while (C) prechordal tissue grafted to the opposite side of the same host embryos was found to be *gsc* positive (n=6). This demonstrates that our RHP grafts do not routinely include prechordal tissue. Caudal head process grafts were also assessed for contamination with adjacent (*paraxis*-expressing) paraxial tissues. (D) CHP grafts placed in contact with the extra-embryonic epiblast for 4 hours did not contain *paraxis*-expressing cells, while grafts of adjacent paraxial tissue were found to express this gene when transplanted from stage 6+-7 donor embryos (n=4), although paraxial tissue grafted from stage 6 embryos did not express *paraxis* (not shown, 4/4). These findings indicate that paraxial tissue is not routinely included with CHP grafts derived from HH6+-7 and that such tissue is unlikely to contaminate CHP derived from HH6 embryos. Scale Bar, 50 μm.

to form a neural tube in the majority of cases (12/14), while CHP induces a neural tube less frequently (3/18) and fails to induce any recognisable neural tissue in 9/18 cases (Fig. 4F; Table 1). These findings suggest that RHP is a stronger source of neural inducing signals than CHP.

The small size of other amniote embryos (such as the mouse) makes it difficult to separate axial and paraxial mesoderm (Ang and Rossant, 1993; Ang et al., 1994). As these tissues are more easily separated in the chick embryo we next assessed whether the paraxial mesendoderm (PMe) adjacent to RHP and CHP also possess neural inducing activity. Mesendoderm from either region does not induce neural tissue in chick host embryos, as assessed by morphological criteria observed in sections (see Table 1 and Fig. 4G,H). As we did not detect a morphological response to these grafts in the extraembryonic epiblast we also assayed for the induction of an early pan-neural marker, *Sox-2* (Streit et al., 1997). This gene was not induced in response to caudal PMe (n=4; Fig. 4I,J) and nor were any of the regional markers assessed (*Sax-1*, 0/2, *Krox-20*, 0/2 and *Engrailed* 0/2, data not shown). These findings therefore suggest that signals

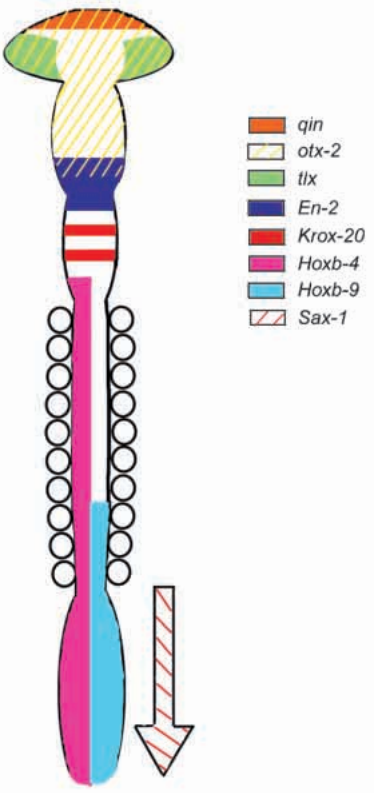

Fig. 2. Panel of region-specific molecular markers. Eight molecular markers were used to identify distinct rostrocaudal domains of the CNS. *qin*, expressed in the rostral forebrain (Chang et al., 1995); *tlx*, expressed in the caudal forebrain (Yu et al., 1994); *otx2*, expressed in the forebrain and rostral midbrain (Bally-Cuif et al., 1995); En-2, expressed in the posterior midbrain/rhombomere 1 of the hindbrain (Patel et al., 1989); *Krox-20*, expressed in hindbrain rhombomeres 3 and 5 (Wilkinson et al., 1988); *Hoxb-4*, expressed in the posterior hindbrain and spinal cord, with an anterior-most boundary at rhombomere 6/7 (Sasaki et al., 1990); *Hoxb-9*, expressed in the posterior spinal cord, with an anterior-most boundary at somites 8/9 (corresponding to C3) (Zeltser et al., 1996); *Sax-1*, expressed transiently in the prospective spinal cord with the anterior-most boundary at the level of the last forming somite (represented by hatched arrow) (Spann et al., 1994).

sufficient to induce new neural tissue in the extraembryonic epiblast of the avian embryo emanate from axial, but not paraxial mesendoderm.

Rostral and caudal head process induce discrete regions of the CNS in competent epiblast

The regional character of the neural tissue induced by rostral and caudal head process in the extraembryonic epiblast was assessed using a panel of region-specific molecular markers (Fig. 2). RHP most frequently induces the expression of the metencephalic markers *Engrailed 2* (12/14 cases) and *Fgf-8* (4/5 cases) and in only one case elicited expression of the forebrain marker *tailless* (1/15 cases; Fig. 5A). RHP also induces hindbrain tissue, as shown by the expression of *Krox-20*, but does not elicit expression of the more caudal markers *Hoxb-4*, *Sax-1* or *Hoxb-9* (Fig. 5A,B-H). Thus, in general, RHP induces neural tissue expressing a restricted range of rostrocaudal neural markers indicative of the formation of the midbrain/anterior hindbrain and including the isthmus region of the CNS.

In contrast, when CHP induced morphologically identifiable neural tissue (assessed in sections, see Figure legend 5A) this was most frequently of posterior hindbrain/anterior spinal cord character, indicated by the

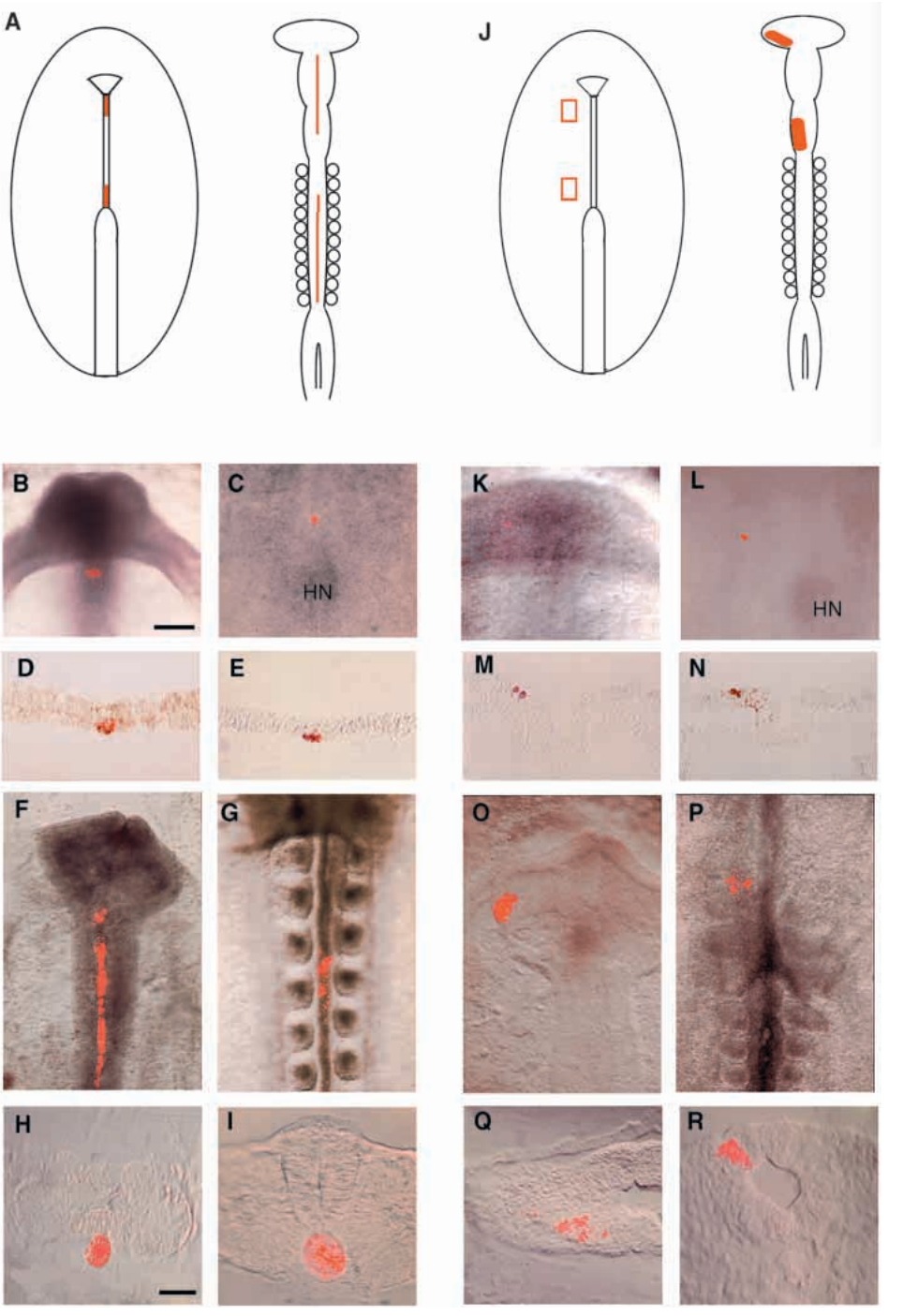

Fig. 3. The fates of the head process and overlying neuroepithelium. (A) Summary diagram showing DiI-labelling of the RHP and CHP at HH6-7 and subsequent distribution of DiI at HH10-11. Groups of 2-5 cells were labelled with DiI (within the regions blocked in red) n values for each position are given in the text. Time 0 images of cells labelled at HH6-7 in the (B) RHP and (C) CHP. A subset of these embryos (*n*=3) were photoxidised immediately and sectioned to confirm that axial mesendoderm was labelled with this method; (D) transverse section (TS) of RHP, t=0; (E) TS of CHP, t=0. (F) RHP maps to the notochord underlying the caudal diencephalon/midbrain and anterior hindbrain at HH10-11, in this embryo two initial injections were made either end of the rostral red block (see A), while CHP maps to the notochord beneath the spinal cord between somites 3-12 (G), which is shown in sections of RHP (H) and CHP (I). (J) Summary diagram showing the region in the neural plate (outlined in red) where groups of 2-5 cells were DiI-labelled at the level of rostral and caudal HP at HH6-7 and the subsequent distribution of DiI at HH10-11. Cells were DiI-labelled in rostral (K) and caudal (L) neural plate at HH6-7. A subset of embryos were photoxidised immediately and sectioned to confirm that only neuroepithelial cells had been labelled, e.g. RNP (M; *n*=2) and CNP (N; *n*=2). RNP maps to the diencephalon in the region of the developing optic lobes (O) while CNP maps to the posterior hindbrain (P), also shown in sections of RNP (Q) and CNP (R). HN, Hensen's Node. Scale Bar, 200 μm (B,C,D,E,F,G,K,L,M,N,O,P); 50 μm (H,I,Q,R).

Table 1. Neural inducing abilities of rostral head process (RHP), caudal head process (CHP) and paraxial mesendoderm (PMe) at head-fold stage

	Neural tube	Neural plate	Raised epithelium	No induction
RHP	12/14 (86%)	2/14 (14%)	0/14	0/14
CHP	3/18 (17%)	4/18 (22%)	2/18 (11%)	9/18 (50%)
PMe	0/6	0/6	0/6	0/6

induction of *Hoxb-4* and *Sax-1* and the failure to elicit *Hoxb-9* (Fig. 5A,I-M). Older CHP grafts taken from HH8-9 embryos failed to induce neural tissue and could therefore not be assessed for the ability to elicit *Hoxb-9* (*n*=5; data not shown). Occasionally CHP did elicit expression of more rostrally expressed neural markers such as *Krox-20* and Engrailed 2 (Fig. 5A). This indicates that the CHP also induces only a discrete region of the CNS and that this region is distinct from that induced by the RHP.

Rostral and caudal head process do not induce region-specific genes in neural plate explants

To investigate the ability of rostral and caudal head process to provide regional information to the neural plate, we isolated the neural plate or combined it with its underlying head process. In these experiments explants of neural plate and head process were both derived from quail donors and grafted into HH3-3+ chick host embryos. The QCPN antibody was combined with in situ hybridisation for region-specific genes to distinguish gene expression in neural plate explants from that in ectopic neural tissue that might be induced in the chick host (see Figs 1 and 6)

Rostral neural plate (RNP) explants grafted alone express *tlx* and *otx-2* (Fig. 6A-C) but not more caudal markers (*En-2*, *Krox-20*, *Hoxb-4* and *Sax-1*). This tissue also does not express *qin*, a marker of rostral forebrain and together these findings identify our rostral neural plate explants as caudal forebrain tissue (see Fig. 2; Fig. 6A-D). When RNP is recombined with RHP, *tlx* expression is restricted to two discrete patches in a

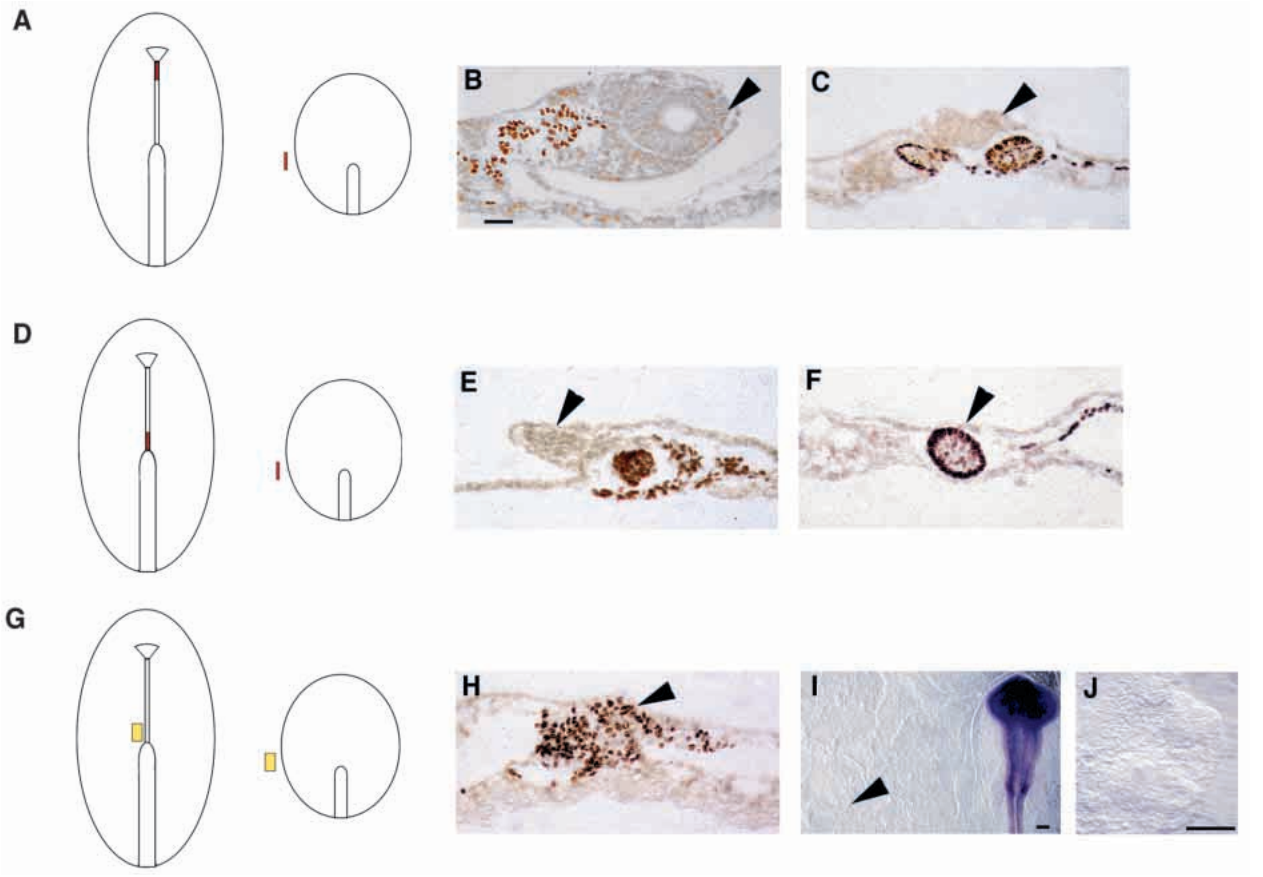

Fig. 4. Comparison of the neural inducing abilities of rostral head process, caudal head process and paraxial mesendoderm. Quail-derived grafts were juxtaposed with host chick extraembryonic epiblast. Following incubation grafts were distinguished from host tissue using immunocytochemistry to detect the quail-specific antigen QCPN (visualised with a peroxidase-conjugated secondary antibody; brown perinuclear staining) in sections. Ectopic neural structures were then assessed by their morphology. (A) RHP was grafted to an ectopic site. (B) RHP most frequently induces a neural tube (arrowhead). (C) CHP (see below) most frequently induces a neural plate (arrowhead). (D) CHP was grafted to an ectopic site. (E) CHP can also induce a region of raised epithelium (arrowhead). (F) Overlying extra-embryonic epiblast cells (arrowhead) did not form neural structures in response to CHP in half of the cases examined (see Table 1). (G) Paraxial mesendoderm was grafted to an ectopic site. (H) Paraxial mesendoderm does not induce neural tissue (arrowhead) or (I) ectopic expression of the early neural marker *Sox-2*, which was only detected in host neuroepithelium. (J) High magnification of grafted paraxial mesendoderm confirming the absence of *Sox2* expression. Scale bar, 50 μm (B-H); 150 μm (I-J).

pattern resembling its normal dorsoventral distribution within the forebrain (Yu et al., 1994), *otx2* expression remains uniform throughout the RNP when grafted alone or in combination with the RHP (Fig. 6A,C,F). However, the presence of RHP does not elicit expression of markers of more caudal neural tissue in the RNP explant (Fig. 6A,G). This result is surprising given that the RHP is a source of signals able to induce neural tissue expressing more caudal genes (*En-2* and *Krox-20*; see Discussion).

Caudal head process inhibits expression of rostral neural genes

Grafts of caudal neural plate (CNP) alone express *Hoxb-4*, *Krox-20* and in one instance *En-2* (1/6), but not *Sax-1* or *Hoxb-9* and therefore differentiate into hindbrain/anterior spinal cord (Fig. 6H-J). Strikingly, CNP no longer expresses *Krox-20* when grafted together with underlying CHP, however *Hoxb-4*, which is expressed more caudally than *Krox-20*, is still observed (Fig. 6H,K,L). As *Krox-20* is expressed throughout the dorsoventral extent of the neural tube its absence is unlikely to result from the complete ventralisation of neural plate explants (see below). Further, the molecular identity of CNP in these recombinations is consistent with the regional character of the neural tissue, which the CHP normally comes to underlie later in development (see Fig. 3) (i.e. beneath *Hox-b4* but not *Krox-20* expressing CNS). This suggests that during normal development the CHP provides inhibitory signals that refine the rostrocaudal character of overlying neural tissue.

The ability of CHP to inhibit more rostral neural character was tested further by combining RNP with CHP (both quail derived) and grafting them together into the extraembryonic epiblast of a HH 3-3+ chick embryo (Fig. 7A-F). While *tlx* is

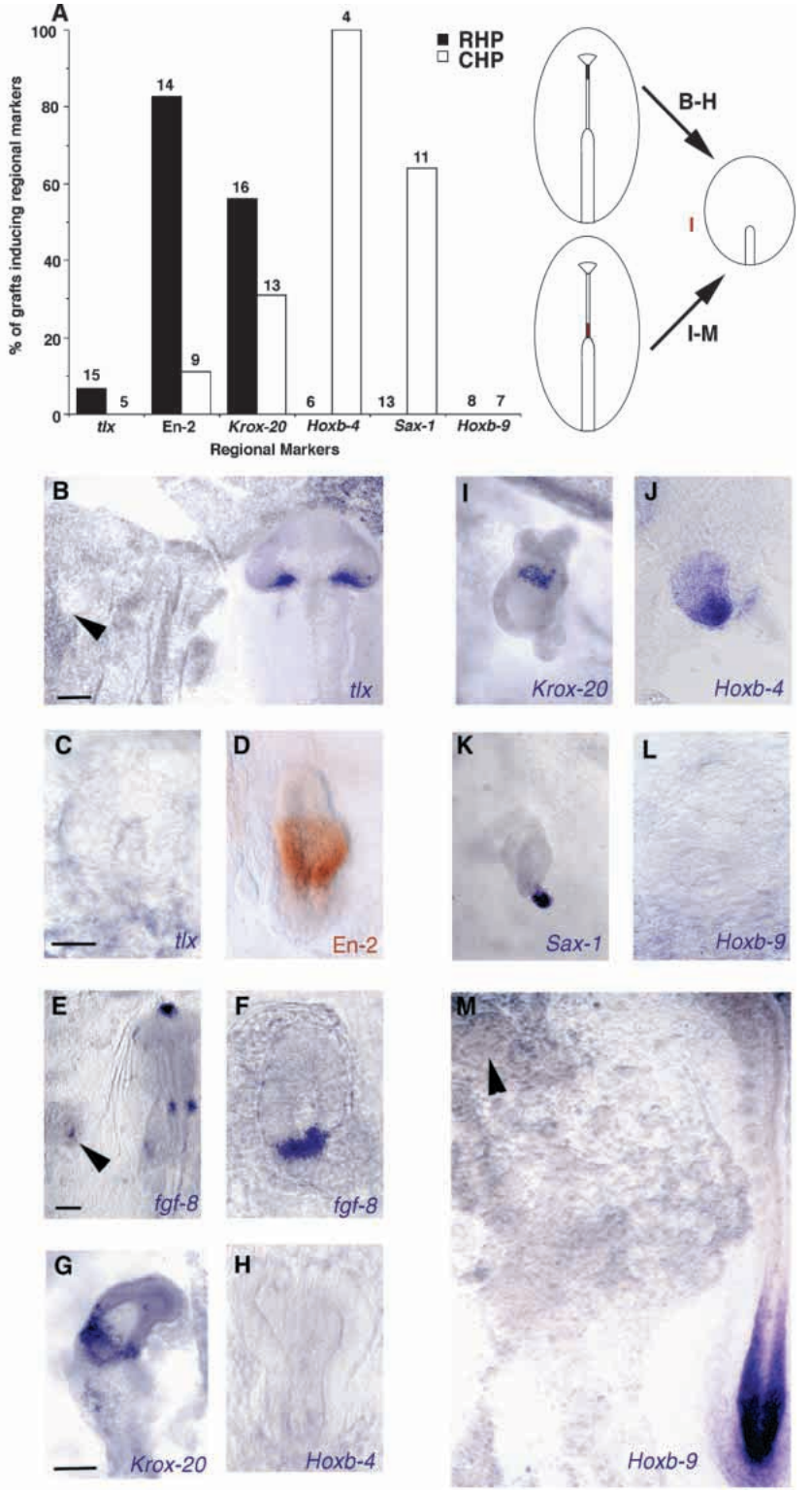

Fig. 5. Induction of region-specific markers by rostral head process and caudal head process. (A) Percentage of RHP and CHP grafts inducing six region-specific molecular markers (described in Fig. 2). Expression of all markers was assessed following whole-mount in situ hybridisation unless stated otherwise and *n* values indicate percentage of induced neural structures expressing regional markers; consistent with data from QCPN labelled grafts, CHP did not induce neural tissue (assessed in sections) in approximately 50% of cases, (for example, while only 4/9 CHP grafts induced expression of *Hoxb-4*, the 5 non-expressing cases also failed to form any morphologically identifiable neural tissue). Thus, only grafts that induced ectopic neural structures are included in this histogram. (B) RHP (arrowhead) does not induce neural tissue expressing *tlx* but this gene is detected in the host embryo. (C) High magnification of graft site in B showing absence of *tlx*. (D) RHP induces neural tissue expressing En2, detected by immunocytochemistry and revealed with a peroxidase-conjugated secondary antibody. (E) Neural tissue induced by RHP also expresses a patch of *Fgf8* (arrowhead) which in combination with En2 expression in this tissue is indicative of the isthmus region of the midbrain/hindbrain. (F) High magnification of graft site in E showing the induction of neural tissue expressing *Fgf-8*. (G) Neural tissue induced by RHP also expresses *Krox-20*. (H) Neural tissue induced by RHP does not express *Hoxb-4*. (I) CHP induces neural tissue expressing *Krox-20*, (J) *Hoxb-4* and (K) *Sax-1*. (L) Neural tissue induced by CHP however, does not express *Hoxb-9*. (M) Whole-mount showing the position of graft site in L with respect to the *Hoxb-9*-positive host embryo. Scale bar, 200 μm (B,M); 300 μm (E); 100 μm (C,D,F,H,L); 150 μm (G,I-K).

expressed in isolated RNP (see Fig. 6), it is not detected in 8/11 cases when this tissue is combined with CHP. To further investigate this result we combined in situ hybridisation for *tlx* with immunocytochemistry for a notochord marker (Brachyury). This revealed that in two cases where *tlx* was expressed in RNP explants, the CHP was no longer close to the neural plate, suggesting that contact between these two tissues is required to inhibit *tlx* expression (the remaining 9 cases showed direct contact of RNP/CHP tissue) see Fig. 7A,B. Explants of RNP also express *otx2*, we therefore next assessed whether CHP also affects the expression pattern of this gene. In 7/8 cases *otx2* expression was reduced and in one case absent in RNP/CHP combinations (compare Figs 6C with 7C,D; also see 7I). These findings indicate that signals from

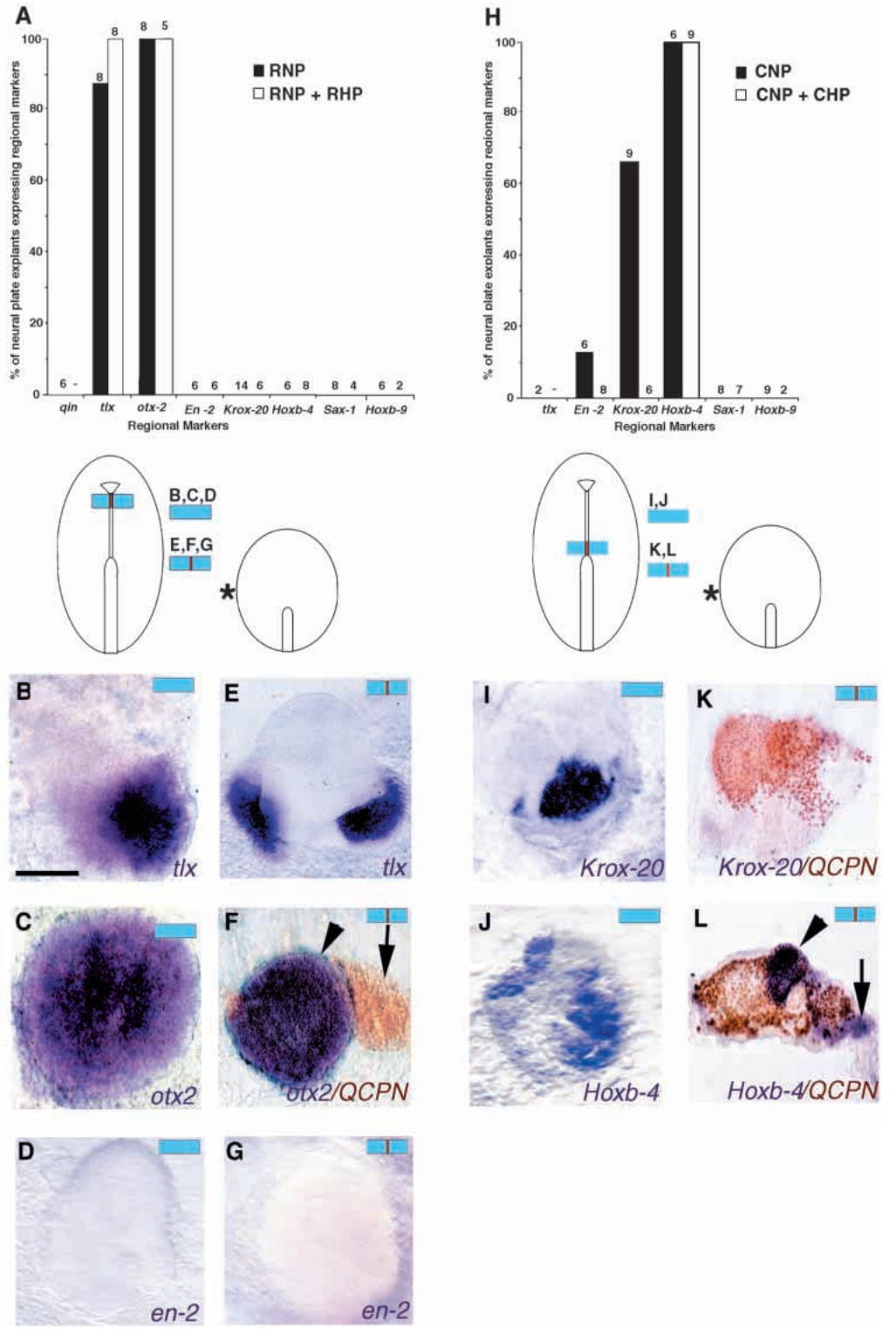

Fig. 6. Expression of region-specific markers in rostral neural plate (RNP) or caudal neural plate (CNP) explants alone or following recombination with underlying axial mesendoderm. Percentage of RNP explants expressing a panel of region-specific markers, grafted alone or following recombination with RHP. Gene expression was scored following whole-mount in situ hybridisation; *n* values are indicated above each bar. (A) RNP explants alone or in combination with RHP expresses only forebrain markers. (B) *tlx* is expressed in a single broad domain in RNP explants. (C) *otx2* is expressed throughout the RNP explant. (D) RNP explants do not express the midbrain/hindbrain marker *en-2*. (E) Expression of *tlx* is restricted to two discrete patches following recombination with underlying RHP. (F) RNP (arrowhead) expresses *otx2* when recombined with RHP (arrow). Both tissues were quail derived (QCPN positive) as this allowed us to distinguish RNP explants from neural tissue induced in the chick host, which might also express *otx2*. (G) *en-2* is not expressed in RNP explants following recombination with RHP. (H) Percentage of CNP explants expressing six region-specific markers, grafted alone or following recombination with CHP; *n* values above each bar. CNP explants do not express *En2* and *Krox20* in the presence of the CHP. (I) CNP explants express the hindbrain marker *Krox-20*. (J) CNP explants express *Hoxb-4*. (K) CNP explants grafted following recombination with CHP do not express *Krox-20*. QCPN expression (brown labelling) was used to distinguish quail neural plate explants from neural tissue that might be induced in the chick host and could possibly express *Krox-20*. (L) A section through *Hoxb-4*-expressing, QCPN-positive CNP explants (arrowhead) grafted following recombination with CHP. In this case *Hoxb-4* is also detected in host chick cells which are not recognised by the QCPN antibody (arrow). Scale bar, 100 μm (B-G; I-L).

the CHP can inhibit gene expression in rostral as well as caudal neural plate.

We next tested whether the failure to express *tlx* and the down regulation of *otx2* in the RNP/CHP combinations is accompanied by the induction of more caudally expressed neural genes. Despite its ability to induce de novo expression of *Sax-1*, *Krox-20* and *Hoxb-4* in extraembryonic epiblast, CHP fails to induce these genes in the QCPN-positive explants of RNP (*Krox-20*, 0/5; *Hoxb-4*, 0/9; *Sax-1*, 0/2) (Fig. 7E,F), indicating that in this assay inhibition of rostral genes can be separated from the induction of caudal genes. These findings therefore suggest that RNP when combined with CHP has a diencephalic character as indicated by the expression of *otx2* in the absence of *tlx*.

Inhibitory signals are not provided by newly emerged head process at HH5

As the RHP is older than the newly emerged CHP at HH6-7 (see Introduction) it is possible that the inhibitory activity of CHP reflects its age. We therefore tested whether newly emerged RHP at HH5 can also inhibit gene expression in RNP explants (derived from HH6-7). We find that *tlx* is still expressed in RNP following combination with HH5 RHP (*n*=5/5; data not shown), demonstrating that these inhibitory signals are a distinct property of newly emerged head process at HH6-7.

Caudal head process vs paraxial mesendoderm

Recent reports (Bang et al., 1997; Itasaki et al., 1996; Muhr et al., 1997) have shown that paraxial mesendoderm (before and after overt segmentation) is a source of caudalising signals in the chick embryo. We have therefore compared the regionalising abilities of caudal paraxial mesendoderm (CPMe) with those of the CHP described above. In all cases CPMe did not alter the expression pattern of *tlx* (4/4, Fig. 7G) or *otx2* (4/4, not shown) in the RNP. The effects of caudal paraxial tissue were also compared with those of the rostral paraxial mesendoderm. In all RNP/RPMe combinations expression of *tlx* (2/2, Fig. 7H) and *otx2* (7/7, Fig. 7I) remained uniform. We also further assessed whether CPMe induces expression of caudal neural genes *Krox-20* and *Hoxb-4* in RNP. In all cases we find that these tissues do not induce caudal markers in RNP (*Krox 20*, 0/6, Fig. 7J and *Hoxb-4*, 0/6 Fig. 7K). Together, these results show that caudal axial but not paraxial mesendoderm can inhibit the expression of forebrain-specific genes and that neither paraxial nor axial mesendoderm can induce expression of caudal neural genes in this assay.

Inhibition of rostral neural markers is not due to ventralisation of neural explants

One interpretation of the absence of *tlx* expression and the down regulation of *otx2* in RNP/CHP combinations is that the CHP is a stronger source of ventralising signals than the RHP and that the neural explant has acquired a completely ventral character. To test this possibility we assessed RNP/CHP combinations for (a) expansion of the ventral, floor plate marker sonic hedgehog (*shh*) (Riddle et al., 1993) and (b) the presence of a marker of dorsal neural tissue, *slug* (Nieto et al., 1994). We find that the expression

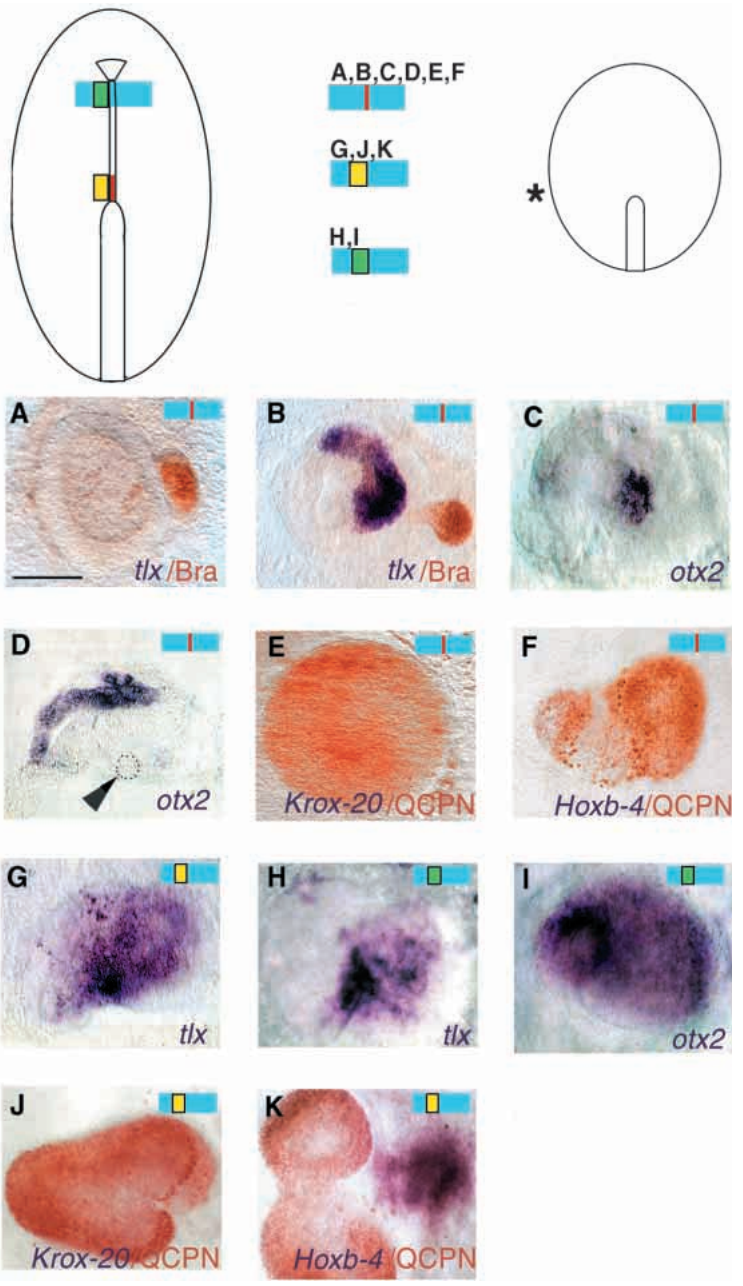

Fig. 7. Effects of caudal head process and paraxial mesendoderm on rostral neural plate. (A) Expression of *tlx* is lost in RNP explants recombined with the CHP (CHP is identified here using immunocytochemistry for a Brachyury-specific antibody visualised following reaction of a peroxidase-conjugated secondary antibody). (B) In the few cases in which CHP (brown cells) did not remain in direct contact with the RNP *tlx* expression was detected in this neuroepithelium. (C) *otx2* expression is down regulated in the RNP when juxtaposed to CHP. (D) Transverse section of explant similar to that in C, shows position of CHP (arrow) with respect to expression of *otx2* in RNP. (E) *Krox-20* is not induced in quail-derived RNP juxtaposed with CHP (neural plate explants in E,F,J,K appear brown following labelling with QCPN and the reaction of a peroxidase-conjugated secondary antibody). (F) *Hoxb-4* is also not expressed in RNP combined with CHP. (G) *tlx* expression is not lost in RNP explants when combined with caudal paraxial mesendoderm (C- PMe) or rostral paraxial mesendoderm (R- PMe) (H). (I) *otx2* expression in RNP explants remains uniform when combined with R- PMe. (J) RNP explants combined with C-PMe do not express *Krox-20*. (K) *Hoxb-4* is also not induced in quail-derived RNP combined with C- PMe. Scale bar, 150 μm.

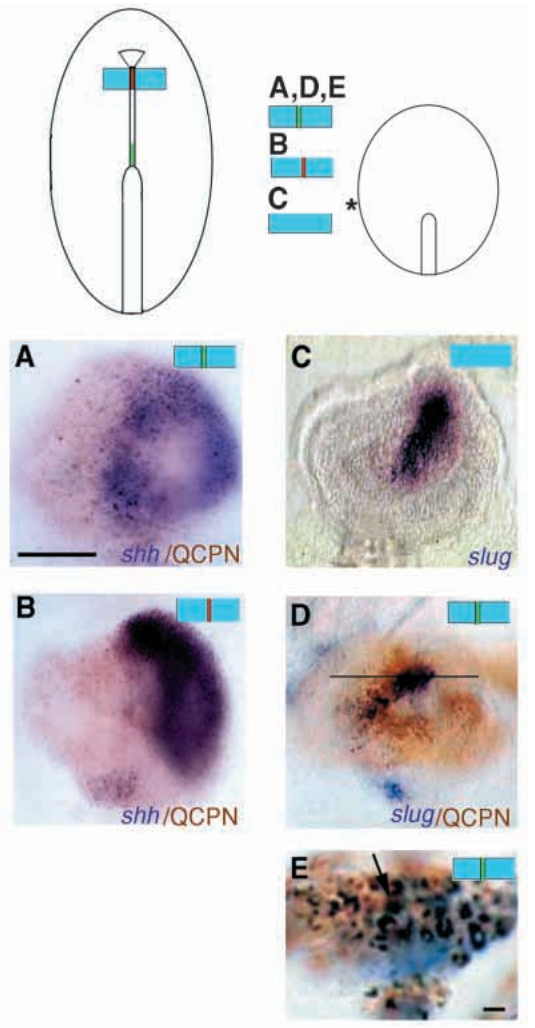

Fig. 8. Both dorsal and ventral cell types are present on neural plate explants when combined with axial mesendoderm. (A) *shh* expression in RNP after re-combination with CHP. (RNP explants (A,B,D,E) are identified here with the QCPN antibody detected with a peroxidase-conjugated secondary antibody and appear brown). (B) *shh* expression in RNP after combination with RHP. (C) *slug* expression in RNP explants. (D) *slug* expression in RNP when combined with CHP. (E) Transverse section of D (horizontal line), arrow indicates a cell double labelled with QCPN and *slug*. Scale bar, 150 μm (A-D); 10 μm (E).

pattern of *shh* is similar in RNP combined with RHP (*n*=5) and CHP (*n*=4). Patches of *shh* expressing cells were detected, but these never extended throughout explants (Fig. 8A,B). The dorsal neural tube marker *slug* was also found to be expressed in RNP/CHP combinations (*n*=2/3; Fig. 8C-E) further supporting the view that the RNP does not become completely ventralised in these experiments. Together, these findings suggest that the inhibition of rostral neural genes cannot be simply ascribed to ventralising signals provided by CHP.

DISCUSSION

We have established the fates and relative movements of the rostral and caudal head process and their overlying neural plate and have assessed the neural inducing and regionalising abilities of these discrete populations of axial mesendoderm. We show that the neural inducing strength of head process decreases along its length and that this activity is not shared with adjacent paraxial mesendoderm. Rostral and caudal head process can induce neural tissue with distinct rostrocaudal characteristics in competent epiblast, however, they cannot induce expression of region-specific markers in neural plate explants, which we show have already acquired some rostrocaudal character. Thus, at headfold stages during normal development discrete regions of axial mesendoderm do not establish rostrocaudal pattern in overlying neuroepithelium. However, caudal head process can inhibit the expression of genes characteristic of neural tissue rostral to the region of the CNS that it normally comes to underlie. Strikingly, this inhibitory activity is not shared with paraxial tissues and is not accompanied by the expression of the caudal neural markers assessed.

Regional differences in neural inducing activity in the mesendoderm

The first axial mesendoderm cells to emerge from the node between HH stages 4+ and 5 are a mixed cell population consisting of both prechordal and prospective head process cells. This cell population has previously been shown to possess neural inducing activity (Izpisúa-Belmonte et al., 1993; Foley et al. 1997; Pera and Kessel, 1997). The prechordal tissue quickly separates from the head process cells as they move rostrally, followed by the emergence of the more caudal head process by HH6 (see Fig. 1). Here, we demonstrate that RHP (located just caudal to the prechordal mesendoderm at HH6-7) is a more potent neural inducer than CHP, which consists of tissue that emerged from the node about 6h after the RHP. This finding is consistent with the weaker neural inducing ability of nodes at HH5-6 (see Storey et al., 1992) and suggests that it is the prospective rostral head process cells in the node that are the source of neural inducing signals (see Selleck and Stern, 1991; Storey et al., 1995). Further, we show that flanking paraxial mesendoderm is not able to induce neural tissue in this embryo, although somitic tissue is reported to possess this activity in other vertebrates (Hemmati-Brivanlou et al., 1990; Ang et al., 1993), thus localising neural inducing signals to the rostral axial tissues. These findings indicate that this tissue may play a role in the induction of neural tissue during normal development. However, it is clear, from node ablation studies in the chick (Grabowski, 1956; Darnell et al., 1992; Spann et al., 1994) that such tissue can form in the absence of axial mesendoderm. A similar conclusion can be drawn from the presence of neural tissue in mice lacking *HNF3β*, which fail to form a morphological node and notochord (Ang et al., 1993; Weinstein et al., 1994). Thus, during normal development the neural inducing signals provided by the head process may simply reinforce earlier signals provided by the node and may perhaps serve to carry these signals to rostral regions of the embryo (see Knoetgen et al., 1999).

Rostral and caudal head process can induce different regions of the CNS

Our DiI labelling study shows that rostral and caudal head process at HH6-7 contribute to regions of notochord that at stage 10-11 lie beneath the caudal diencephalon/anterior

hindbrain, and anterior spinal cord (adjacent to somites 3-12), respectively. When rostral and caudal head process are grafted in contact with competent extraembryonic epiblast, each induces a specific range of regional markers, which broadly correspond to the region of prospective nervous system that they underlie at the time of excision. This finding is consistent with Hara's (1961, 1978) earlier observations that relied solely on morphological criteria. Using a range of molecular makers we have refined Hara's findings. While Hara's rostral head process frequently induced forebrain characteristics, our experiments demonstrate that this tissue induces only midbrain/hindbrain regions of the CNS. The 1/15 case in which the forebrain marker *tailless* was induced is most likely explained by the inadvertent inclusion of prechordal tissue, which has recently been shown to induce forebrain markers (Foley et al., 1997; Pera and Kessel, 1997). While Hara had difficulty separating spinal cord from hindbrain tissues induced in response to CHP, we find that this tissue induces hindbrain but also expression of the early and transient spinal cord marker *Sax-1* as well as *Hoxb-4*, a marker of a region of the posterior hindbrain and spinal cord. Strikingly, *Hoxb-9*, which is expressed in more posterior spinal cord is not induced by CHP, again suggesting that there is a tight correlation between the rostrocaudal level from which head process is derived and the regional character of neural tissue induced by it. This finding raises a question that we have posed previously (see Mangold, 1933; Storey et al., 1992): are there as many regionalising signals as there are regions of the CNS? These findings show that different rostrocaudal levels of the head process are sources of distinct regionalising signals that could contribute to the establishment of specific rostrocaudal regions of the CNS. However, here we have tested the ability of discrete regions of head process to induce neural tissue with particular regional characteristics, but during normal development the head process emerges beneath a region that has already received neural inducing signals (reviewed in Streit and Stern, 1999) and which may have already acquired rostrocaudal pattern. We therefore next assessed the regional specificity of rostral and caudal neural plate explants at HH6-7 and then subsequently assessed whether signals from the underlying rostral and caudal head process were responsible for the regulation of region-specific genes.

Rostrocaudal character is established in HH6-7 neural plate explants

We found that explants of rostral and caudal neural plate (at HH6-7) go on to express complementary sets of region-specific markers in the absence of underlying mesendoderm. Explants of RNP come to express the forebrain markers *tlx* and *otx2*, but not markers of the rostral forebrain or caudal midbrain, *qin* and *En2* respectively. This fate is consistent with the results of our DiI-labelling study which shows that the neural plate overlying the RHP at HH6-7 will come to lie rostral to the axial mesendoderm and contribute to the caudal forebrain (Fig. 3O). This rostral movement of the neuroepithelium has been previously described in mammalian embryos (Morriss-Kay and Tuckett, 1987) and our lineage analysis also confirms a similar recent study of rostral neural plate in the avian embryo (Dale et al., 1999). Similarly, explants of CNP come to express *Krox-20* and *Hoxb-4*, a fate also consistent with our lineage tracing study, which identifies neural plate overlying the CHP at HH6-7 as prospective hindbrain. These findings indicate that by HH6-7 the neural plate has already acquired some rostrocaudal pattern and that it does not depend on signals from underlying mesendodermal tissue to maintain distinct regional characteristics; a finding consistent with the results of node ablation experiments (Darnell et al., 1992). However, as there is a relative shift in the rostrocaudal position of the neuroepithelium and the underlying axial mesendoderm during development, it is possible that signals from axial mesendoderm serve to refine rostrocaudal character. We therefore next assessed whether rostral and caudal head process could alter patterns of region-specific gene expression in neural plate explants.

Rostral head process does not induce midbrain/hindbrain markers in RNP

While RNP explants come to express a gene combination characteristic of the caudal forebrain, the RHP normally comes to underlie the prospective midbrain/hindbrain. When these two tissues are recombined RNP explants maintain their forebrain identity (as assessed by the expression of *tlx* and *otx-2*) and do not express more caudal markers (including *En2* and *Krox-20*). This is despite the fact that RHP is a source of signals that can induce (de novo) neural tissue characteristic of the isthmic (*En2* and *Fgf8*) and hindbrain (*Krox-20*) regions of the CNS. The failure to induce expression of midbrain/hindbrain markers in prospective forebrain explants is therefore surprising. This finding may reflect a loss of competence in response to these signals by headfold stages, although it has been shown that older caudal diencephalic tissue is competent to express *En2* in response grafts of En2-expressing tissue or FGF signals (Martinez et al., 1991; Crossley et al., 1996; Shimamura and Rubenstein, 1997). It might therefore be that such signals are not effective when provided by vertically apposed tissues (see below). We have also found that RHP does not alter the gene expression profile of caudal neural plate explants (*Hox-b4* expression is maintained n=4; data not shown). It is clear, however, that the rostral neural plate can respond to other signals provided by the RHP, as *tailless* expression which is maintained in these recombinations, becomes restricted to two discrete domains within the neural plate explant. The patterning of *tlx* expression suggests that RHP signals do impose dorsoventral pattern, consistent with previous reports (Tanabe and Jessell, 1996; Dale et al., 1997) and not inconsistent with a more recent study showing that this tissue can regulate the rostrocaudal character of ventral midline cells (Dale et al., 1999).

Caudal head process can inhibit expression of rostral neural genes

In contrast with the RNP/RHP combinations in which we did not observe changes in the panel of region-specific markers expressed in RNP, the regional character of CNP is altered by prolonged interaction with CHP. While explants of CNP come to express a set of genes characteristic of the hindbrain (*Krox-20* and *Hoxb-4*), the CHP comes to underlie a more caudal region of the CNS. Strikingly, when the CNP is recombined with the CHP, *Krox-20* expression is now not detected, while *Hoxb-4* is present in the CNP explant. This change in the combination of genes expressed in CNP suggests that it has acquired a more caudal character when combined with CHP,

and this character correlates well with that of the neural tube which the CHP comes to underlie later in development. As both *Krox-20* and *Hoxb4* are normally expressed throughout the dorsoventral extent of the neural tube they should both still be detected if the CHP acts just to impose dorsoventral pattern on the CNP. These findings therefore suggest that the CHP is a source of signals that can refine the rostrocaudal character of the neuroepithelium. We therefore next tested whether the CHP could also inhibit rostral gene expression in explants of the rostral neural plate.

Remarkably, RNP/CHP combinations also lead to the loss of rostral characteristics as manifest by the failure to express *tlx* and the down regulation of *otx2*. It is interesting that CHP can inhibit *tlx* but only down regulate *otx2* expression in the RNP. This may be because CHP signals inhibit the initiation of *tlx* expression, whereas *otx2* is already expressed in RNP explants at the time of excision. It is also unlikely that this result is due to the stronger ventralising signals provided by the CHP, as we detected expression of the dorsal neural tube marker (*slug*) in these combinations and found the ventral marker (*shh*) remained confined to discrete regions of explants. These results therefore show that the CHP can also inhibit rostral genes in RNP and further support the proposition that during normal development this axial tissue provides signals that refine rostrocaudal character.

Why are caudal neural genes not induced by axial and paraxial mesendoderm in this assay?

It is surprising that the loss of rostral gene expression in RNP explants combined with CHP was not associated with the induction of the caudal neural markers *Hoxb-4* and *Krox 20*. This finding and recent experiments identifying paraxial mesoderm as a source of caudalising signals (Bang et al., 1997; Itasaki et al., 1996; Muhr et al., 1997; Woo and Fraser, 1997) led us to assess whether adjacent paraxial mesendoderm can induce caudal neural genes in these rostral explants. We found that the caudal paraxial mesendoderm also did not induce *Hoxb-4* or *Krox-20* in RNP. The failure to elicit *Krox-20* expression in this tissue at this stage however, confirms the findings of Muhr et al. (1997) who also failed to detect *Krox-20* or *En-2* in prospective forebrain explants cultured in vitro with paraxial mesoderm.

So why do we fail to induce expression of caudal neural genes in this assay? One possibility is that these signals need to be provided as neural tissue is being induced, as has recently been suggested by Kolm et al. (1997). We have already raised the possibility that regionalising signals may also be required to be presented within the plane of the neuroepithelium. Indeed, it has recently been shown that prospective diencephalon at HH7+ is competent to express the caudal marker *Hoxb-4* if it is transplanted into *Hoxb-4*-expressing neural plate (Grapin-Botton et al., 1997). On the other hand, other regions of the CNS can be induced to express *Hoxb-4* in response to paraxial tissue (Itasaki et al., 1996) demonstrating that vertical apposition of mesendoderm and neuroepithelium can elicit expression of caudal neural genes. This suggests that homeogenetic signals provided by the neuroepithelium might be different from those presented by mesendoderm and that the competence to respond to these different signals may vary along the rostrocaudal length of the CNS. It is also possible that juxtaposition of RNP and CHP leads to the acquisition of a discrete regional character that falls between the neural markers used in this study or may even generate a nonsense code (see Pownall et al., 1996; Grapin-Botton et al., 1997). However, this does not apply to CNP/CHP combinations and we predict that during normal development signals provided by the CHP act by repressing expression of rostral genes as the caudal CNS is laid down.

Candidate inhibitory signals

A similar inhibitory activity in posterior mesendoderm (consisting of both axial and paraxial tissues) has been reported in the mouse embryo, which restricts *otx-2* expression to its later rostral domain (Ang et al., 1994). Here we find that this inhibitory activity is not shared with paraxial tissues or newly emerged RHP at HH5, indicating that it is a specific property of caudal axial mesendoderm. Inhibitory signals derived specifically from axial mesoderm also appear to play a role in the restriction of *Hoxb-1* expression in the *Xenopus* embryo (Poznanski and Keller, 1997). In the mouse, the inhibitory activity of posterior mesendoderm can be mimicked by retinoic acid (Ang et al., 1994) and this molecule can also inhibit *otx2* expression in the chick (Bally-Cuif et al., 1995). Retinoic acid is detected at high levels in the head-fold stage node (Chen et al., 1992) and is therefore also likely to be present in the caudal head process making it a candidate molecule responsible for the inhibitory activity of the CHP. FGFs may also play a role, as they are also expressed in the head process; FGF4 is present transiently in this structure between HH5-7+ (Shamin et al., 1999) and FGF2 is found in the caudal head process and flanking tissue (Riese et al., 1995). FGFs have been shown to inhibit expression of rostral neural genes as well as having a caudalising effect on the early neural plate (e.g. Rodriguez-Gallardo et al., 1996; Henrique et al., 1997; but see Muhr et al., 1997; KGS unpublished data), however, the role of FGF signalling in patterning the neural plate at head fold stages has yet to be examined in detail (see Shamin et al., 1999). Thus, there are a number of candidate molecules that may inhibit expression of rostral neural genes and it seems likely that combinations of such factors at specific concentrations will be responsible for the activity of the CHP.

Conclusion

These findings clarify the role of head process in the induction and rostrocaudal patterning of the neural tube. Although the RHP is a potent neural inducer it is likely that it serves simply to reinforce earlier node-derived signals. Similarly, while we demonstrate that different levels of head process are able to induce discrete rostrocaudal regions of the CNS in competent epiblast, these pieces of axial mesendoderm do not elicit expression of region-specific genes in neural plate explants derived from head fold stage embryos, which we show have already acquired rostrocaudal pattern. This indicates that discrete regions of axial mesendoderm do not establish this pattern. Our results do however, identify a novel role for the differentiating CHP in refining rostrocaudal identity; by inhibition of genes expressed rostral to the region of the neural tube that it normally comes to underlie. These findings support the view that regionalisation is a stepwise process and our identification of the caudal head process as a source of signal(s) that inhibit rostral neural genes will help to elucidate the molecular mechanisms underlying rostrocaudal specification.

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