

## Capacity to form choroid plexus-like cells *in vitro* is restricted to specific regions of the mouse neural ectoderm

Tim Thomas and Marie Dziadek

Centre for Early Human Development, Monash Medical Centre, Clayton Rd., Clayton, Victoria 3168, Australia

### SUMMARY

Neural ectoderm was dissected from 9.5-day and 8.5-day gestation mouse embryos and divided into forebrain, midbrain, hindbrain and spinal cord regions. Forebrain and hindbrain material from 9.5-day neural ectoderm was further divided into presumptive choroid plexus regions and regions that would normally form nervous tissue *in vivo*. All tissues were plated onto a basement membrane substratum for culture *in vitro*. It was found that explants of neural ectoderm that would normally form choroid plexus *in vivo*, readily differentiated to form choroid plexus-like cells in culture. Cells from hindbrain segments and forebrain regions, which would normally form nervous tissue, also had the potential to differentiate into cells resembling the choroid plexus epithelium in culture, provided that the normal cell-cell interactions were disrupted. Cells from the midbrain neuromeres of 9.5-day embryos, which do not form a choroid plexus *in vivo*, did not form this lineage

*in vitro*. However, cells cultured from the earlier head-fold stage midbrain neural ectoderm could develop into choroid plexus epithelium. There was no evidence that neural ectoderm from the spinal cord had the developmental potential to form choroid plexus epithelial cells at either of these two developmental stages. These studies show that the restrictions in the potential of neural ectoderm stem cells to form different lineages proceeds according to morphological divisions that appear along the anterior-posterior axis during the early stages of brain development. These results suggest that the division of neural ectoderm into segments which contain discrete stem cell populations may be a general feature of the early phase of development of the central nervous system.

Key words: choroid plexus, brain, neural ectoderm, segmentation, cell lineages

### INTRODUCTION

During the development of the vertebrate brain, transverse boundaries appear within the neural ectoderm at the time of neural tube closure giving the brain a segmented appearance (Kallen and Lindskog, 1953; Tuckett et al., 1985). In the developing chick hindbrain the pattern of neuronal differentiation corresponds to morphological segmentation termed rhombomeres (Lumsden and Keynes, 1989). Cell labelling experiments have shown that after these boundaries form, the progeny of cells within different rhombomeres no longer mix and so these segments form distinct cell populations (Fraser et al., 1990; Guthrie and Lumsden, 1991). The pattern of expression of Hox and other regulatory genes in the mouse neural ectoderm coincides with these segmental boundaries (Holland and Hogan, 1988; Kessel and Gruss, 1990; Hunt et al., 1991a,b; Tuggle et al., 1990). These observations have prompted comparisons with *Drosophila melanogaster* development, where segmented patterning of the embryo specifies the adult body plan. However, in the mammalian brain, the extent to which early segmentation of the neural ectoderm dictates subsequent development is unknown. In this study we have analyzed

the differences in the developmental potential of different regions of the embryonic mouse brain by assessing their capacity to differentiate into choroid plexus-like cells.

The choroid plexus epithelium is derived from neural ectoderm and is found in the lateral and third ventricles of the forebrain and in the fourth ventricle of the hindbrain. In most mammalian species the third ventricle choroid plexus is continuous with the lateral ventricle choroid plexus and so may be derived from a single cell population (Netsky and Shuangshoti, 1975). The hindbrain choroid plexus forms from neural ectoderm cells in the roof of the fourth ventricle. In the rat, the events leading to the formation of the choroid plexus first occur in the hindbrain at about 12.5 days of gestation, when the pseudostratified epithelium in the region of the roof of the fourth ventricle becomes a thin layer of columnar epithelial cells. These epithelial cells are much more tightly associated with the underlying mesenchymal tissue via a basement membrane than is the surrounding neural tissue (Thomas et al., 1988). The choroid plexus epithelial cells secrete large quantities of the plasma thyroid hormone transport protein, transthyretin. It has been postulated that this facilitates the transport of thyroxine across the blood-brain barrier (Dick-

son et al., 1987; Schreiber et al., 1990). Transthyretin mRNA can be detected in choroid plexus epithelial cells from the earliest stage that these cells can be distinguished from surrounding neural tissue, and is thus a sensitive marker for the differentiation of this cell type (Thomas et al., 1988).

When fetal mouse choroid plexus epithelial cells are cultured on a reconstituted basement membrane substratum they form hollow spherical vesicles. These cells have tight junctions, apical microvilli, and retain many other features of the choroid plexus epithelium *in vivo* including the production of transthyretin (Thomas et al., 1992). At 9.5 days of gestation, the mouse neural tube is a pseudostratified epithelium consisting almost entirely of undifferentiated cells having a homogeneous morphology (Buse and Krisch, 1987). We have used a culture system to compare the potential of undifferentiated neural ectoderm from various regions of the 8.5-day and 9.5-day mouse brain to differentiate into choroid plexus epithelial cells. We demonstrate that despite the outward homogeneous appearance of the neural ectoderm at these stages, different cell populations can be identified by their ability to differentiate into choroid plexus-like cells. Regions with developmental fates that do not include a choroid plexus lineage *in vivo*, but which readily differentiated along this pathway in culture were identified.

## MATERIALS AND METHODS

### Embryo dissection and cell culture

Embryos were obtained from naturally mated Random Swiss mice. The gestational age was calculated from the middle of the dark cycle preceding the observation of a vaginal plug. Embryos were dissected from pregnant females on days 8.5 and 9.5 of gestation. The neural tube was isolated from each embryo and dissected along the segmental boundaries. Other cell layers were removed from the neural ectoderm layer by incubating tissue pieces in 2.5% pancreatin/0.5% trypsin for 30 minutes at 4°C (Levak-Svajger et al., 1969) and then separating tissue layers using tungsten needles. Tissue was either cultured as explants on a reconstituted basement membrane substratum Matrigel, (Collaborative Research) or dispersed into single cells or small groups of cells by treatment with trypsin/EDTA solution for 3 minutes. Matrigel is a solubilized extract of the mouse Engelbreth Holm Swarm tumor containing collagen IV, laminin, heparan sulphate proteoglycan and nidogen as major components (Kleinman et al., 1986). 150 µl aliquots of Matrigel per well were allowed to gel at 37°C for at least one hour before use. Approximately 5×10<sup>4</sup> neural ectoderm cells were seeded per 16 mm culture well. Most cells attached to the substratum within 24 hours, forming small aggregates. After 10 days of culture in Dulbecco's modification of Eagle's medium supplemented with 10% fetal calf serum (Gibco batch No. 551), neurite outgrowth was usually well advanced and epithelial vesicles were visible. These vesicles developed most rapidly in cultures of cells which normally form choroid plexus epithelia *in vivo*. After 14 days culture, total RNA was purified from cells within each well and northern gel analysis was performed using formaldehyde denaturing gels and hybridization with a rat transthyretin cDNA probe, as described below.

### RNA purification

RNA was purified from cultured cells using a modification of the method of Chirgwin et al. (1979). Briefly, the substratum plus

attached cells were dissolved in 600 µl of 7.5 M guanidine-HCl, 50 mM sodium citrate, 0.25% N-laurylsarcosine, 0.1 M 2-mercaptoethanol, containing 10 µg of *E. coli* RNA as a carrier. This solution was acidified with 40 µl of 1.0 M acetic acid which aided the dissolution of the Matrigel. The RNA was precipitated by the addition of 400 µl of absolute ethanol and pelleted by centrifugation. In order to purify intact RNA it was necessary to repeat the extraction, which was done in the absence of detergent. The pellet was then washed in ethanol and resuspended in 0.1 M sodium acetate and precipitated with 2 volumes of absolute ethanol. The final pellet was resuspended in northern gel loading buffer and RNA species were separated by electrophoresis at pH 7.0 in 1.2% agarose/6.6% formaldehyde gels with buffer circulation as described by Maniatis et al. (1982). RNA was purified from freshly dissected tissue using a similar procedure except that in the first step a ratio of 1 volume of tissue to 10 volumes of buffer was used and the RNA concentration quantitated by absorbance at 260 nm. Gels were stained with ethidium bromide and photographed before RNA was transferred to nitrocellulose filters. RNA fixed to the filters was incubated in hybridization buffer containing 50% formamide at 42°C and a <sup>32</sup>P-labelled cDNA probe coding for rat transthyretin as described previously (Dickson et al., 1985). An IGFII probe was produced by amplification of rat genomic DNA using the polymerase chain reaction. The 10 µg *E. coli* carrier RNA added to each sample prior to extraction allowed the yield of RNA to be assayed. In most cases the yield of *E. coli* RNA, and presumably eukaryotic RNA, were similar for each culture. Experiments where significant differences in the yield of *E. coli* RNA occurred were discarded.

### In situ hybridization

The Matrigel substratum and attached cells were rapidly frozen in OCT low temperature embedding compound (Miles, Naperville) using isopentane cooled on dry ice. Serial 8 µm sections were cut using a Reichert-Jung Frigedocut cryostat. Sections were air-dried onto gelatin-coated microscope slides and fixed in ice-cold 2.5% glutaraldehyde in phosphate-buffered saline for 5 minutes. After fixation, slides were washed in phosphate-buffered saline then dehydrated through graded ethanol and air-dried. If not used immediately, slides were stored at 4°C for 1-2 days in the presence of desiccant.

A synthetic oligonucleotide probe (5'-TTC GGT CAA CTT CTC ATC TGT GGT GAG CCC GTG CAG CTC-3') complementary to rat transthyretin mRNA was used. This probe has been used previously to localize transthyretin mRNA to rat visceral yolk sac and choroid plexus epithelial cells (Fung et al., 1988; Thomas et al., 1988) and also binds specifically to mouse visceral yolk sac endoderm and choroid plexus epithelial cells (data not shown). Control sections incubated with an oligonucleotide complementary to rat  $\alpha_1$  acid glycoprotein mRNA (5'-GTC TGT GGT CTG AAA CTC CCG AAG TTC AAT-3') (Thomas et al., 1989a) did not show any binding to choroid plexus cells above background (data not shown). Sections were hybridized under coverslips with <sup>32</sup>P-end-labelled probes (approximately 5-10 ng of DNA in 20 µl) for 24 hours at 40°C in a buffer consisting of 50% formamide, 0.6 M NaCl, 0.06 M trisodium citrate, 0.1% ficoll, 0.1% bovine serum albumin, 0.1% polyvinylpyrrolidone, 5 mM EDTA at pH 7.3 as described previously (Hudson et al., 1981). Sections were washed under conditions that ensured stability of the oligonucleotide hybrids, which included two washes in 0.6 M NaCl and 0.06 M trisodium citrate at 40°C for 30 minutes with gentle agitation and a final wash in 0.15 M NaCl, 0.015 M trisodium citrate at 40°C for 30 minutes. Sections were then dehydrated by rinsing in increasing concentrations of ethanol. For cellular localization, slides were dipped in Amersham LM-1 liquid emulsion and exposed at 4°C. After development, slides were stained with haematoxylin.

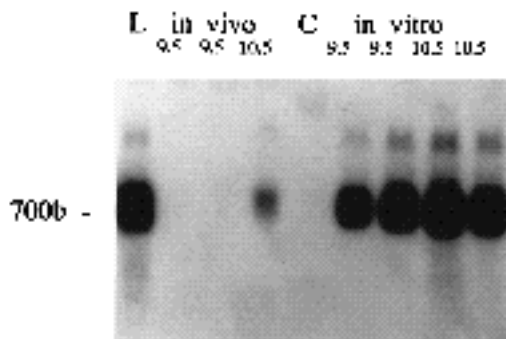
## RESULTS

### In vitro differentiation of the presumptive choroid plexus from the embryonic hindbrain

Total RNA was purified from the hindbrain region of 9.5-day and 10.5-day mouse embryos. No transthyretin mRNA could be detected by northern gel analysis of 15 µg of total RNA from 9.5-day embryos while low levels were detected in RNA purified from 10.5-day embryos (Fig. 1). The first morphological signs of choroid plexus formation are seen in 10.5-day mouse embryos, which is at a similar stage of development as the rat choroid plexus on 12.5 days of gestation reported previously (Thomas et al., 1988). The presumptive choroid plexus region in the roof of the fourth ventricle was dissected from 9.5-day and 10.5-day embryos and cultured as explants on Matrigel substratum for 14 days as described in the Materials and Methods. High levels of transthyretin mRNA were detected in 1-2 µg total RNA purified from these cultures (Fig. 1).

### Comparison of the potential of different regions of the neural tube from 9.5-day embryos to differentiate into choroid plexus cells in vitro

In these experiments, different regions of the neural ectoderm, as shown in Fig. 7, were dissected free of mesenchyme and dispersed into single cells before culture on Matrigel. Differentiation was assessed by northern gel analysis of transthyretin mRNA and by the appearance of spherical epithelial vesicles. The appearance of vesicles formed from 12.5-day fetal choroid plexus epithelial cultured on Matrigel are shown for comparison in Fig. 2A. There were no cultures where vesicles were observed without transthyretin mRNA being detected by subsequent



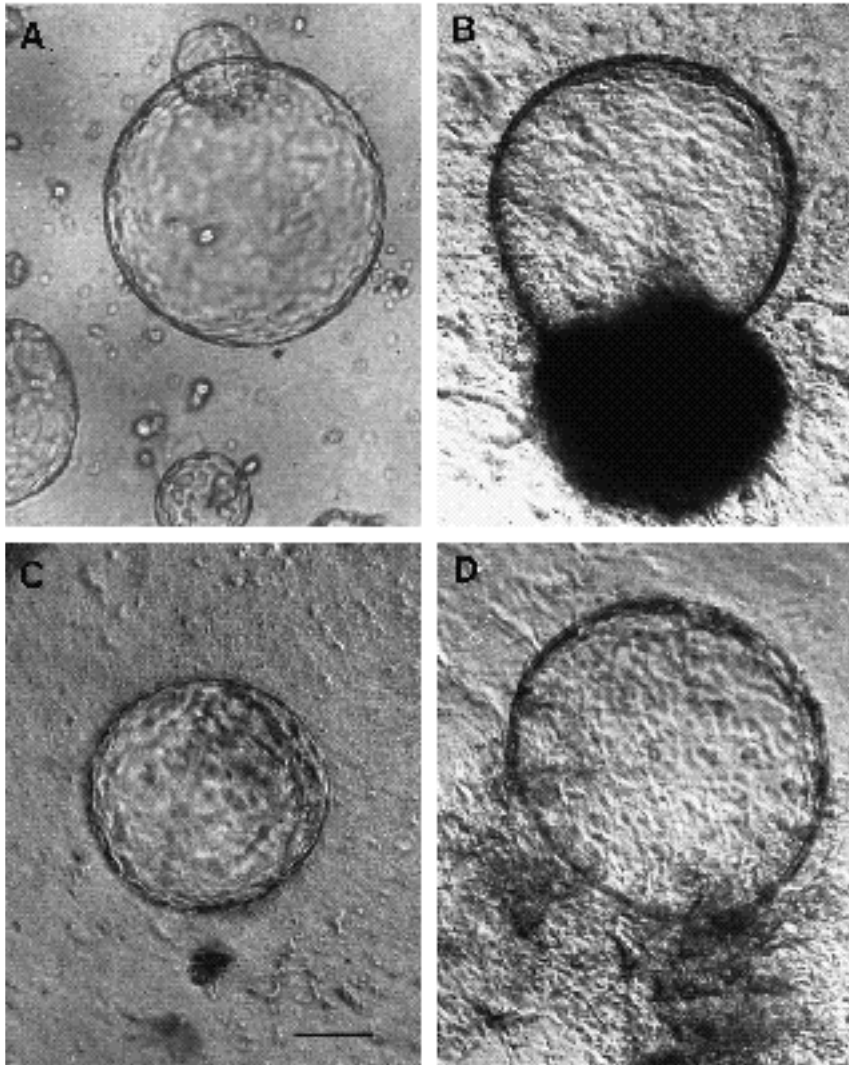
**Fig. 1.** Levels of transthyretin mRNA in the hindbrain in vivo and after culture in vitro. For in vivo samples, RNA was purified from hindbrains dissected from 9.5- and 10.5-day embryos. Transthyretin mRNA levels were assayed by northern gel analysis using 15 µg of 9.5-day hindbrain RNA and 10 µg of 10.5-day hindbrain RNA. For in vitro samples, explants of neural ectoderm dissected from the roof of the hindbrain of 9.5- or 10.5-day embryos was cultured for 14 days, as described in Materials and Methods. Transthyretin mRNA levels were assayed in RNA samples purified from these cultures after addition of 10 µg *E. coli* RNA as carrier. Lanes labelled 'in vitro' contain between 1-2 µg of eukaryotic RNA in addition to *E. coli* RNA. Lane C contains 10 µg of *E. coli* RNA alone. Lane L contains 5 µg of adult mouse liver RNA used for standardization. Duplicate samples contain RNA from separate dissections and cultures. b, bases.

northern gel analysis. In order to analyze any differences in the developmental potential of the different regions of neural ectoderm in the developing brain and spinal cord, the ability of these regions to differentiate into choroid plexus cells was determined.

In the first experiment, neural ectoderm tissue was isolated from the hindbrain segments in which a choroid plexus lineage subsequently forms, but regions of these segments were taken which do not normally contribute to this structure. Thus the floor of the hindbrain, which normally develops exclusively into neural tissue in vivo, was isolated. The development of this region was compared to cell preparations from the adjacent midbrain neuromere which does not contain a choroid plexus in vivo. When the normal cell-cell interactions were disrupted by trypsinization, a substantial percentage of cells from the floor of the fourth ventricle, but not from the midbrain, differentiated in culture to form vesicle structures (Fig. 2C) which were morphologically indistinguishable from those that form from 12.5-day fetal choroid plexus epithelial cells (Fig. 2A) or from explants and dispersed cells from the roof of the fourth ventricle of 9.5-day embryos (Fig. 2B). High levels of transthyretin mRNA were detected in all cultures where vesicles were observed (Fig. 3A).

In the second series of experiments the floor of the fourth ventricle was divided into two halves along the midline, one half of which was cultured as an explant and the other dispersed into single cells. An equivalent number of cells from the roof of the fourth ventricle were also dissected and cultured as explants. The roof of the fourth ventricle explants (ie. choroid plexus primordium) continued to develop in vitro forming epithelial vesicles expressing high levels of transthyretin mRNA (Fig. 3A,B), which are characteristic of fetal choroid plexus cells in culture (Thomas et al., 1992). No vesicles were observed to develop from neural ectoderm explants taken from the floor of the fourth ventricle, where cells differentiated into types that produced extensive neurite outgrowths. No transthyretin mRNA was detected in these cultures (Fig. 3B). As described above, when the normal tissue architecture was disrupted, a large proportion of cells differentiated into epithelial cells forming vesicles. These studies demonstrate the important role of cell-cell interactions in lineage specification in the hindbrain.

In the third series of experiments the developmental potential of neural ectoderm from the forebrain was compared to neural ectoderm from the spinal cord. In the forebrain, transthyretin mRNA is found in the retinal pigmented epithelium of the eye (Cavallaro et al., 1990) as well as in the choroid plexus of the third and lateral ventricles. Thus the telencephalon, from which all cells that normally contribute to the choroid plexus had been removed, was used to determine whether parts of the forebrain that do not normally contribute to the choroid plexus in vivo have the potential to form this structure in vitro. It was found that explants of forebrain choroid plexus primordium readily differentiated in culture to form epithelial vesicles, whereas the remaining telencephalon neural ectoderm differentiated in culture to form vesicles only when normal cell-cell interactions had been disrupted. In contrast, neither vesicles nor transthyretin mRNA were detected in any cultures of spinal



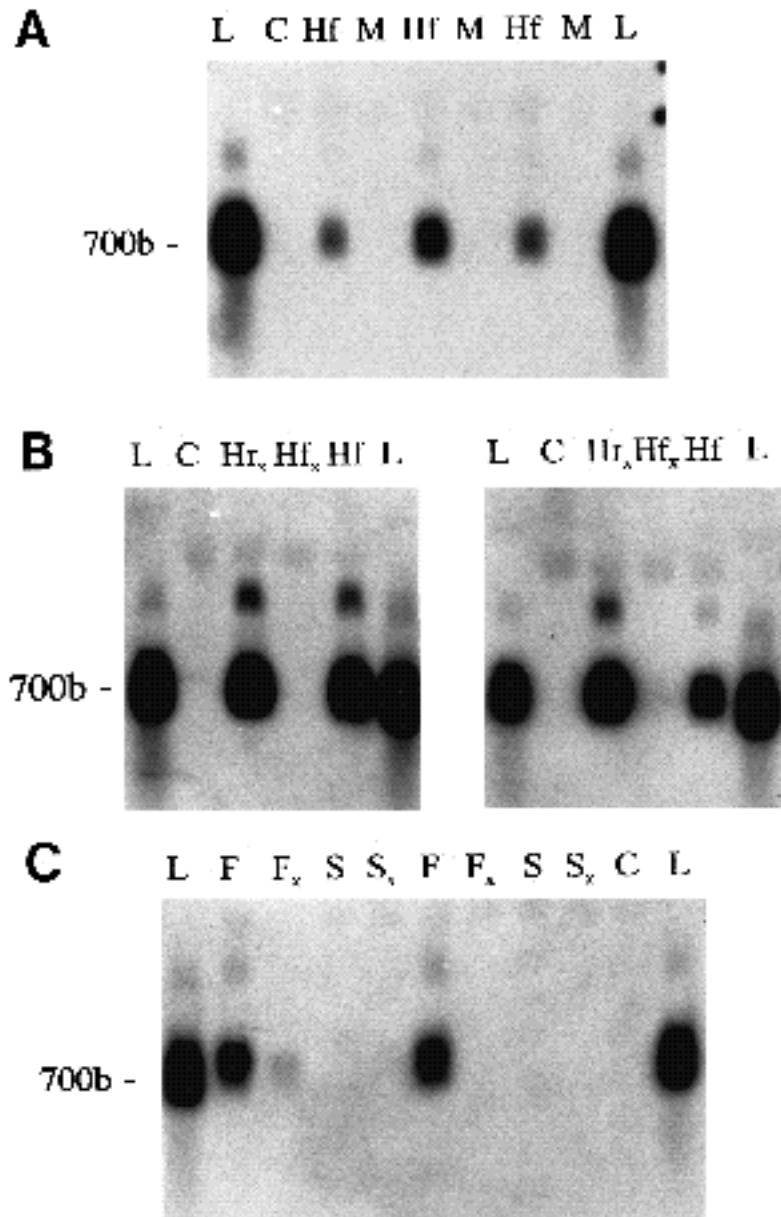
**Fig. 2.** Comparison of vesicles formed from. (A) 12.5-day fetal hindbrain choroid plexus; (B) roof of the fourth ventricle from 9.5-day embryos, cultured as explants; (C) floor of the fourth ventricle from 9.5-day embryos cultured as single cells and (D) telencephalon from 9.5-day embryos co-cultured as single cells with somite mesoderm. Photographs were taken after 14 days in culture and are all at the same magnification. Bar, 100  $\mu\text{m}$ .

cord tissue. These studies proved to be more difficult than those using midbrain and hindbrain neural ectoderm, due to the poor survival of both forebrain and spinal cord neural ectoderm after disruption of normal tissue structure. To eliminate the possibility that in cultures of dispersed cells from forebrain and spinal cord only a specific subset of cells had survived, which were not representative of the whole tissue, the culture system was modified to achieve cell survival rates comparable to hindbrain and midbrain cultures. When neural ectoderm cells from forebrain and spinal cord were co-cultured with somite mesoderm, they preferentially associated with mesoderm cells during culture, and their survival and growth were significantly enhanced.

In cultures of dispersed cells from the telencephalon, co-cultured with somite mesoderm, the majority of cells differentiated to form epithelial vesicles, and high levels of transthyretin were detected in these cultures (Figs 2D, 3C). Explant cultures of telencephalon or spinal cord were viable in culture, and were not co-cultured with mesenchyme. Explants of whole telencephalon did not differentiate into epithelial vesicles, and transthyretin mRNA was not detected. However, in one such explant culture a few small

tissue pieces (less than 200 cells) became detached from the explants. These tissue fragments subsequently formed vesicles, and low levels of transthyretin mRNA were detected (Fig. 3C). These pieces were presumably too small to sustain the cell-cell interactions which in larger explants direct development along different pathways both in vivo and in vitro. When large explants of forebrain, which included the choroid plexus primordium, were cultured, differentiation of epithelial vesicles was observed (not shown) suggesting that the forebrain choroid plexus primordium has similar properties in vitro to the hindbrain choroid plexus primordium. However, cells derived from the telencephalon differed from those of other neural ectoderm regions in their response to the culture system. Extensive neurite outgrowth was seen in explants from other regions of the neural tube, such as from the floor of the fourth ventricle (Fig. 4B), while cells from telencephalon explants differentiated to form migratory cells resembling neuroblasts (Fig. 4B).

No epithelial vesicles or transthyretin mRNA were detected in cultures of spinal cord neural ectoderm, when cells were either dispersed and cultured with somite mesenchyme or cultured as explants. Since somite mesenchyme



**Fig. 3.** The capacity of various parts of the 9.5-day gestation neural tube to differentiate into cells resembling the choroid plexus in vitro. 5  $\mu$ g of adult mouse liver (L) was used as a standard (the size of liver standard mRNA is indicated) and 10  $\mu$ g of *E. coli* RNA (C) was used to assay yield. (A) Transthyretin mRNA levels in triplicate single cell cultures prepared from the floor of the fourth ventricle (Hf) are compared with those in cells from the midbrain (M) after 14 days in culture. (B) The floor of the fourth ventricle was dissected from the hindbrain and divided into symmetrical halves along the midline. One half was cultured as single cells or small groups (Hf) while the other half was cultured as an explant (Hf<sub>x</sub>). Transthyretin mRNA levels in these cultures were compared with levels from explants from the roof of the fourth ventricle (Hr). Duplicate experiments are shown. (C) Transthyretin mRNA levels were compared in cells from the spinal cord primordium cultured as explants (S<sub>x</sub>) or single cells (S) and in forebrain cells from the telencephalon cultured as explants (F<sub>x</sub>) or single cells (F). Single cell preparations were seeded with somite mesoderm in approximately a 2:1 ratio. The basement membrane substratum prevented the mesenchymal cells from proliferating noticeably during the culture period. Duplicate experiments are shown. b, bases.

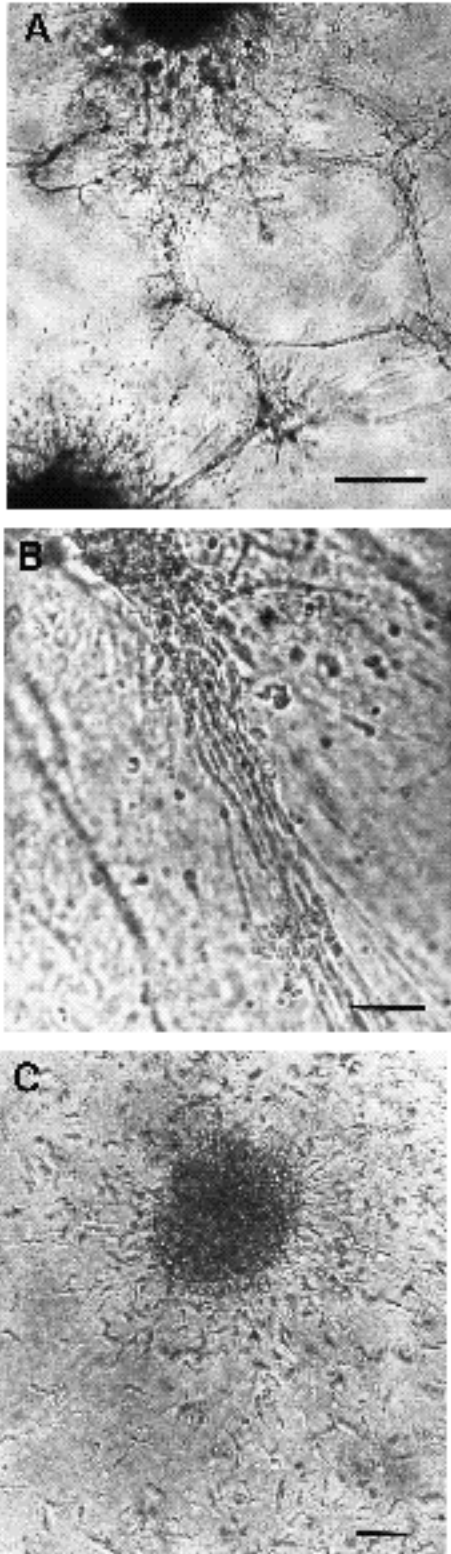
may interact differently with spinal cord than with forebrain neural ectoderm, possibly by inhibiting differentiation into epithelial vesicles, additional studies included co-culture of spinal cord neural ectoderm with mesenchyme separated from the floor of the fourth ventricle, mesenchyme from 11.5-day and 12.5-day choroid plexus and limb bud mesenchyme. In these experiments no epithelial structures were formed, and no transthyretin mRNA was detected, irrespective of the source of mesenchyme (not shown).

In all of the cell cultures described above, transthyretin mRNA was only detected when epithelial vesicles were observed, and was detected even when only 1-2 vesicles were apparent. To determine whether these vesicles were the only structures in these differentiated neural ectoderm cultures that express transthyretin mRNA, in situ hybridization was performed on 9.5-day neural ectoderm cultures. A series of cultures was prepared from the whole hindbrain neural ectoderm, which differentiates in culture to form all

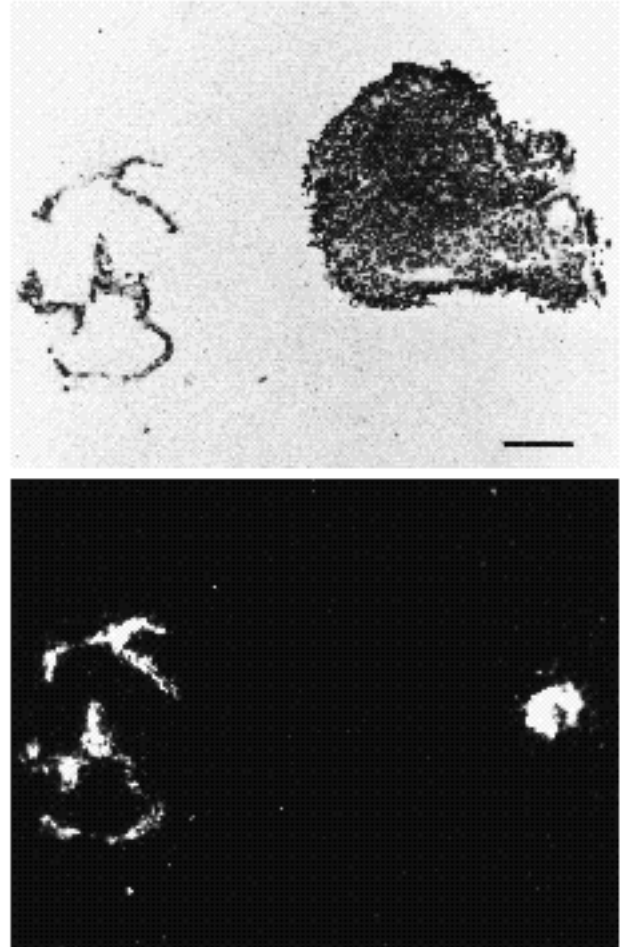
the morphological types observed in the above experiments, including epithelial vesicles and neurite outgrowths. In situ hybridization demonstrated that transthyretin mRNA was localized only to cells forming epithelial vesicles and not to any other multicellular structures (Fig. 5).

#### Comparison of different regions of the head-fold stage neural ectoderm to form choroid plexus-like cells in culture

Morphological boundaries of the spinal cord, hindbrain, midbrain and forebrain can be readily distinguished in the 4-somite embryo at 8.5 days of gestation (Jacobsen and Tam, 1982). To determine whether, at this stage, any neural ectoderm had the capacity to differentiate into choroid plexus-like epithelial cells in culture, these regions were dissected free of mesoderm and other ectoderm and cultured on Matrigel. Single trypsinized cells did not remain viable in culture and so explants were simply cut into

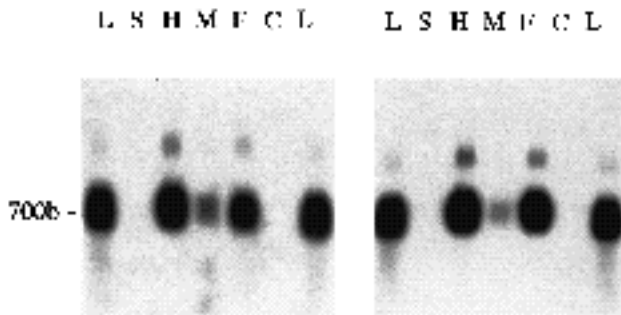


**Fig. 4.** Comparison of explant cultures of the floor of the fourth ventricle and telencephalon dissected from 9.5-day embryos. Extensive neurite outgrowths forming a network between explants are seen in cultures of the floor of the fourth ventricle (A), and at a higher magnification in (B). In contrast, large numbers of migratory cells resembling neuroblasts are seen in explants of telencephalon (C). Explants were cultured for 10 days as described in Materials and Methods. Bars, 100  $\mu$ m (A), 20  $\mu$ m (B, C).



**Fig. 5.** In situ hybridization of a section from 9.5-day hindbrain neural ectoderm cells after 14 days in vitro. The section was incubated with a  $^{32}$ P-labelled transthyretin oligonucleotide probe as described in Materials and Methods. Silver grains are only seen (dark-field optics, lower panel) in association with epithelial vesicles (light-field optics, upper panel). Bar, 100  $\mu$ m.

smaller pieces prior to culture for 10-14 days. No differentiation into choroid plexus-like vesicles was observed in cultures of spinal cord neural ectoderm, whereas vesicles formed very readily in cultures of hindbrain and forebrain neural ectoderm and high levels of transthyretin mRNA were detected (Fig. 6). In midbrain cultures very small pieces of tissue failed to survive, whilst in larger explants extensive neurite outgrowth was observed. However in all midbrain cultures some pieces of ectoderm formed vesicles and low levels of transthyretin mRNA were detected (Fig. 6). These pieces were presumably large enough to survive in culture but too small to differentiate normally. Only in one case out of seven were epithelial structures observed in association with explants which showed outgrowth of neurites, although this was common in hindbrain and forebrain cultures. These results suggest that when the normal cell-cell interactions are disturbed in the midbrain, at least some cells have the potential to differentiate into choroid plexus-like cells. Although transthyretin mRNA was detected in all cultures of brain neural ectoderm, a clear



**Fig. 6.** Comparison of transthyretin mRNA levels in cultures of spinal cord primordium (S), hindbrain (H), midbrain (M) and forebrain (F) dissected from 8.5-day embryos. In each duplicate experiment tissue from eight 4-6 somite embryos was cultured as small pieces. Lanes L contain 5 µg adult liver RNA for standardization. Lane C contains 10 µg *E. coli* RNA used as a carrier during purification of RNA from cell cultures. Between 1-2 µg eukaryotic RNA was purified from each culture. b, bases.

pattern in the propensity of different regions of neural ectoderm from the head-fold stage to differentiate into cells expressing the transthyretin gene was evident (Fig. 7).

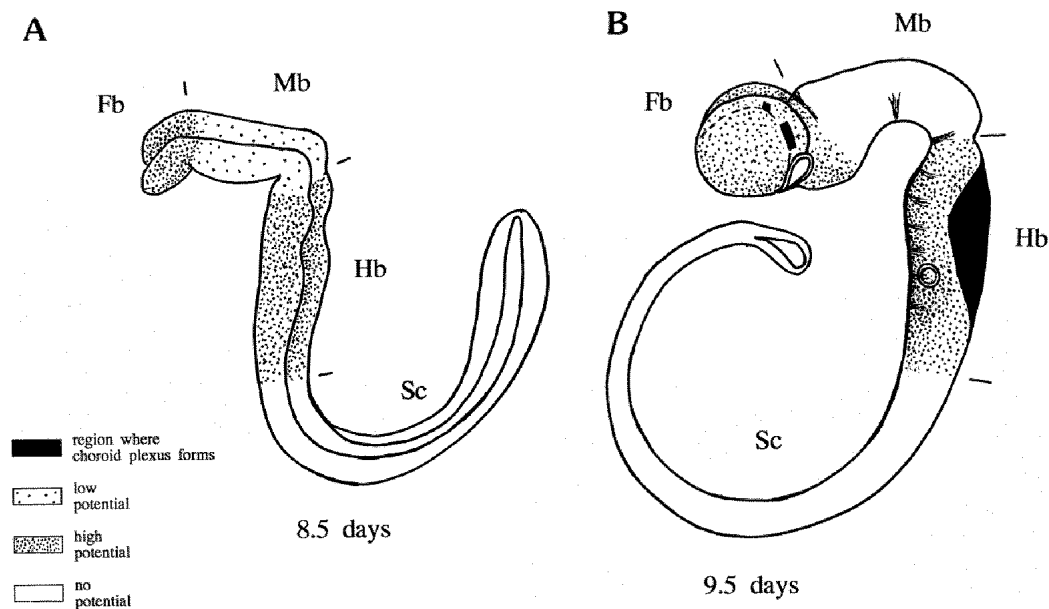
#### Analysis of transthyretin gene expression in cultured fetal retinal pigmented epithelial cells

The retinal pigmented epithelium, which is derived from forebrain neural ectoderm has also been shown to express the transthyretin gene (Cavallaro et al., 1990). In order to determine whether differentiation of this cell type in our cultures is contributing to the formation of vesicles and production of transthyretin mRNA, we evaluated the development of retinal pigmented epithelial cells in our culture system. Retinal pigmented epithelial cells were isolated

from 12.5-day mouse embryos and cultured either as explants or as single cells on Matrigel, as described for the preceding experiments. No vesicle structures were observed to form in these cultures over a period of 14 days. Northern gel analysis showed that the level of transthyretin mRNA produced by these cultures was at least two orders of magnitude less than that in the same quantity of RNA purified from choroid plexus primordium that had differentiated in culture (Fig. 8). The choroid plexus expresses the IGFII gene throughout development (Beck et al., 1987) and so can be used as another marker. No hybridization to IGFII mRNA in the retinal pigmented epithelium could be distinguished above background by *in situ* hybridization (Cuthbertson et al., 1989). When the filter shown in Fig. 8 was incubated with an IGFII probe, no hybridization to RNA from cultured retinal pigmented epithelial cells could be detected, whereas when the filter shown in Fig. 3A was incubated with an IGFII probe, IGFII mRNA could be detected only in cultures shown to contain transthyretin mRNA (data not shown). We conclude that the neural ectoderm cells which differentiate in culture to express the transthyretin gene in the experiments described above, more closely resemble choroid plexus epithelial cells than retinal pigmented epithelial cells.

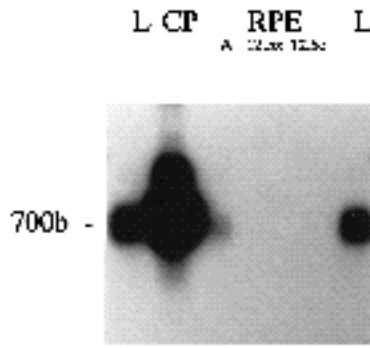
#### DISCUSSION

In this paper we show that cells resembling the choroid plexus epithelium can differentiate in culture from neural ectoderm isolated from regions of the neural tube that do not contribute to the choroid plexus *in vivo*, provided that the normal cell-cell interactions are disrupted. We have demonstrated that at the headfold stage, all regions of the neural ectoderm which subsequently form the brain have



**Fig. 7.** Diagram showing the relationship between different parts of the neural ectoderm at 8.5 and 9.5 days of gestation. At 8.5 days, the neural ectoderm is a sheet of cells which is continuous with the surface ectoderm which will form epidermis and other structures. The outline of ectoderm destined to form forebrain (Fb), midbrain (Mb), hindbrain (Hb) and spinal cord (Sc) at the 4-somite stage is shown. By 9.5 days of gestation the neural tube has closed except at the posterior end. Division of midbrain ectoderm into two neuromeres and hindbrain ectoderm into

rhombomeres is visible at this stage. The regions where the choroid plexus develops *in vivo* are shown in black. The potential of various parts of the neural ectoderm to differentiate into cells resembling the choroid plexus in culture is shown by stippling.



**Fig. 8.** Transthyretin mRNA in fetal retinal pigmented epithelial cells in culture. Retinal pigmented epithelial cells were dissected from 12.5-day fetuses and cultured either as explants (RPE 12.5x) or after dispersion into single cells (RPE 12.5c). RNA was purified from these cultures and

transthyretin mRNA levels assayed by northern gel analysis. Each lane contains 1–2  $\mu\text{g}$  of RNA. Lanes L contain 5  $\mu\text{g}$  of adult liver RNA standard, lane CP contains 4  $\mu\text{g}$  of adult choroid plexus RNA and lane RPE A contains 4  $\mu\text{g}$  of adult retinal pigmented epithelium RNA. b, bases.

the potential to form a choroid plexus lineage, while regions developing into spinal cord do not. After the closure of the neural tube, neural ectoderm isolated from the midbrain does not have the capacity to form choroid plexus cells in this culture system. However, neural ectoderm from the forebrain and hindbrain, including regions which in vivo would exclusively form neuronal and glial lineages, have the potential to differentiate into the choroid plexus. Since the potential to form a choroid plexus epithelium exists in cells that would normally not differentiate along this pathway, this data suggests that these cells have this potential as a consequence of the way in which cell lineages become assigned in the neural ectoderm. These studies also demonstrate that different regions of the neural ectoderm interact differently with the culture environment, although morphologically these regions appear similar. We interpret this to mean that the early stages in differentiation of neural ectoderm results in the formation of distinct stem cell populations along the axis of the neural tube. We believe this is evidence for a model of brain development in which the potential to form a given fully differentiated cell type such as the choroid plexus epithelium is initially possessed by large segments of the neural ectoderm, and as these are subdivided into smaller segments this potential is progressively restricted to specific segments and then to regions within segments.

Recent studies provide persuasive evidence that segmentation is an integral part of brain development in vertebrates. The pattern of neuronal differentiation coincides with morphological segmentation of the hindbrain into rhombomeres (Lumsden and Keynes, 1989). Cell labelling experiments have shown that cells confined by rhombomere boundaries in the chick hindbrain do not intermix after formation of these boundaries (Fraser et al., 1990). Cells within different rhombomeres express a different set of regulatory genes, in particular *Hox* genes (Holland and Hogan, 1988; Hunt et al., 1991a; Murphy et al., 1989; Wilkinson et al., 1989a,b), which presumably reflects the different developmental potential of different segments (Hunt et al., 1991b). Expression of these genes appears to be uniform within the segments where it occurs, suggesting that cells within these segments may have a uniform phenotype

(Puschel et al., 1990). Evidence also exists for the formation of segments in the forebrain (Puelles et al., 1987). The expression of the mouse homologue of the *Drosophila* gene *distalless* is confined by morphological boundaries in the mouse forebrain that are analogous to those described in the chick (Price et al., 1991).

The selective expression of transcription factors is thought to be important in specifying regional developmental programmes. However, it is not yet clear how different patterns of gene expression are directly involved in the specification of different cell lineages arising from the neural ectoderm. Since the *Hox7* gene is expressed at much higher levels by the choroid plexus primordium than by surrounding neural tissue from day 11.5, it has been suggested that this gene may be involved in specifying the choroid plexus (Mackenzie et al., 1991). However, at this stage the transthyretin gene is already expressed by the mouse choroid plexus (Fig. 1). At 9.5 days of gestation, the *Hox7* gene is expressed throughout the brain, including the midbrain (Mackenzie et al., 1991). Since the midbrain lacks the potential to form the choroid plexus and since *Hox7* expression is not specifically localized to regions where the choroid plexus subsequently develops, it seems unlikely that the *Hox7* gene is involved in the earliest events specifying the choroid plexus, but may be involved in its further growth and differentiation.

Evidence from the amphibian system suggests that ectoderm is initially 'dorsalized', giving it a tendency to form forebrain structures. Subsequent interaction with underlying mesenchyme then leads to the formation of caudal structures (Nieuwkoop, 1952; Toivonen and Saxen, 1967; Yamada, 1990). The results summarized in Fig. 7 imply that if a similar two-step process occurs in mouse the transformation to form caudal structures will first establish spinal cord and brain lineages. The brain ectoderm, whose capacity to form the choroid plexus is consistent with a forebrain specification, is subsequently divided into midbrain, hindbrain and forebrain regions. Indirect evidence suggests that the hindbrain forms first but at this time cells can still move from midbrain to forebrain (Tuckett and Morriss-Kay, 1985). The results presented in this paper suggest that in the mouse, division of the headfold into forebrain, midbrain and hindbrain is incomplete in the 4-somite embryo on day 8.5 of gestation, but is complete one day later on day 9.5.

Analyses of immortalized cell lines and brain cells in primary culture have demonstrated the importance of cell-cell interactions and environmental influences on the development of cell types within the central nervous system (reviewed by McKay, 1989). Thus, while the early processes associated with segmentation may specify the developmental options for neural ectoderm cells in a given region, their actual differentiation is contingent on external conditions. Our experiments have shown that disruption of the normal tissue architecture changes the normal differentiation pathway of neural ectoderm. It has been shown that the fate of cells after the neural tube is closed is greatly influenced by their position relative to the floor plate (Yamada et al., 1991; Hirano et al., 1991). The mechanism proposed involves the formation of a dorsal-ventral gradient, possibly involving retinoic acid (Yamada et al., 1991). It is likely that disruption of the normal positional rela-



tionships between cells may prevent the duplication of the molecular events that occur in vivo and so allow alternative pathways of differentiation in culture.

The mechanisms that control choroid plexus differentiation in vivo cannot be inferred from this study. The choroid plexus differentiates earlier than other parts of the brain (Thomas et al., 1989b) and choroid plexus epithelial cells may simply represent one of the most likely differentiated states in a tissue culture environment where the normal cues for differentiation are either absent or altered. The experiments described in this paper do not eliminate the possibility first suggested by Birge (1962) that choroid plexus formation is induced by a subpopulation of mesenchymal cells in vivo. It is possible that extracellular factors produced by mesenchymal cells, such as growth factors and extracellular matrix components, regulate the differentiation of the choroid plexus in vivo, and that these same factors are present in the culture system.

A feature of choroid plexus development in vivo is that during transformation from a pseudostratified epithelium to a columnar epithelium, the cells forming the early choroid plexus are more tightly associated with the underlying basement membrane than are adjacent neuroepithelial cells (Thomas et al., 1988). The data presented here raises the possibility that cell-matrix interactions influence the developmental pathway of neural ectoderm cells and coordinate the differentiation of the choroid plexus without direct participation of the mesenchyme. The extracellular matrix has been shown to play a role in the development of other areas of the brain at specific stages. Laminin promotes the differentiation of neural/glial stem cells into neurons (Drago et al., 1991), and has also been shown to induce amphibian retinal pigmented epithelial cells to differentiate into neurons (Reh et al., 1987). At an earlier stage of amphibian development the extracellular matrix appears to prevent the premature differentiation of ectoderm to neural ectoderm (Grunz and Tacke, 1990).

The results described in this study demonstrate that cells positioned in a region where the choroid plexus normally develops have the potential to differentiate into this cell type even though their normal developmental fate is to form neuronal, glial and ependymal lineages. These results suggest that the processes of differentiation first lead to the formation of cell populations with different developmental potential along the anterior-posterior axis. Subsequently, differences in the developmental potential between dorsal and ventral cells in the neural tube are established, by a process in which cell-cell interactions appear to be important.

This work was supported by a project grant from the Australian Research Council (to G. Schreiber and M. D.) and a National Health and Medical Research Council postdoctoral fellowship (T. T.). We are grateful to Peter Hudson for providing oligonucleotide probes, Elizabeth Stadler for photography and Alison Couper for typing the manuscript.

## REFERENCES

Beck, F., Samani, N. J., Penschow, J. D., Thorley, B., Tregear, G. W. and Coghlan, J. P. (1987). Histochemical localization of IGF-I and IGF-II mRNA in the developing rat embryo. *Development* **101**, 175-184.

- Birge, W. J. (1962). Induced choroid plexus development in the chick metencephalon. *J. Comp. Neurol.* **118**, 89-95.
- Buse, E. and Krisch, B. (1987). The mouse neural plate as starting material for studying neuronal differentiation *in vitro*. *Anat. Embryol.* **175**, 331-340.
- Cavallaro, T., Martone, R. L., Dwork, A. J., Schon, E. A. and Herbert, J. (1990). The retinal pigmented epithelium is the unique site of transthyretin synthesis in the eye. *Invest. Ophthalmol. Vis. Sci.* **31**, 497-501.
- Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J. and Rutter, W. J. (1979). Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* **18**, 5294-5299.
- Cuthbertson, R. A., Beck, F., Senior, P. V., Haralambidis, J., Penschow, J. D. and Coghlan, J. P. (1989). Insulin-like growth factor II may play a local role in the regulation of ocular size. *Development* **107**, 123-130.
- Dickson, P. W., Howlett, G. J. and Schreiber, G. (1985). Rat transthyretin (prealbumin): Molecular cloning, nucleotide sequence, and gene expression in the liver and brain. *J. Biol. Chem.* **260**, 8214-8219.
- Dickson, P. W., Aldred, A. R., Menting, J. G. T., Marley, P. D., Sawyer, W. H. and Schreiber, G. (1987). Thyroxine transport in the brain. *J. Biol. Chem.* **262**, 13907-13915.
- Drago, J., Nurcombe, V. and Bartlett, P. F. (1991). Laminin through its long arm E8 fragment promotes the proliferation and differentiation of murine neuroepithelial cells in vitro. *Exp. Cell Res.* **192**, 256-265.
- Fraser, S., Keynes, R. and Lumsden, A. (1990). Segmentation in the chick hindbrain is defined by cell lineage restrictions. *Nature* **344**, 431-435.
- Fung, W.-P., Thomas, T., Dickson, P. W., Aldred, A. R., Milland, J., Dziadek, M., Power, B., Hudson, P. and Schreiber, G. (1988). Structure and expression of the rat transthyretin (Prealbumin) gene. *J. Biol. Chem.* **263**, 480-488.
- Grunz, H. and Tacke, L. (1990). Extracellular matrix components prevent neural differentiation of disaggregated *Xenopus* ectoderm cells. *Cell. Diff. Develop.* **32**, 117-124.
- Guthrie, S. and Lumsden, A. (1991). Formation and regeneration of rhombomere boundaries in the developing chick hindbrain. *Development* **112**, 221-229.
- Hirano, S., Fuse, S. and Sohal, G. S. (1991). The effect of the floor plate on pattern and polarity in the developing central nervous system. *Science* **251**, 310-313.
- Holland, P. W. H. and Hogan, B. L. M. (1988). Expression of homeobox genes during mouse development: a review. *Genes Dev.* **2**, 773-782.
- Hudson, P., Penschow, J., Shine, J., Ryan, G., Niall, H., and Coghlan, J. (1981). Hybridization histochemistry: The use of recombinant DNA as a homing probe for tissue localization of specific mRNA populations. *Endocrinology* **108**, 353-356.
- Hunt, P., Whiting, J., Muchamore, J., Marshall, H. and Krumlauf, R. (1991a). Homeobox genes and models for patterning in the hindbrain and bronchial arches. *Development Supplement 1*, 187-196.
- Hunt, P., Gulisano, M., Cook, M., Sham, M.-H., Faiella, A., Wilkinson, D., Boncinelli, E. and Krumlauf, R. (1991b). A distinct Hox code for the branchial region of the vertebrate head. *Nature* **353**, 861-864.
- Jacobsen, A. G. and Tam, P. P. L. (1982). Cephalic neurulation in the mouse embryo analyzed by SEM and morphometry. *Anat. Rec.* **203**, 375-396.
- Kallen, B. and Lindskog, B. (1953). Formation and disappearance of neuromy in *Mus musculus*. *Acta Anat.* **18**, 273-282.
- Kessel, M. and Gruss, P. (1990). Murine developmental control genes. *Science* **249**, 374-379.
- Kleinman, H. K., McGarvey, M. L., Hassell, J. R., Star, V. L., Cannon, F. B., Laurie, G. W. and Martin, G. R. (1986). Basement membrane complexes with biological activity. *Biochemistry*, **25**, 312-318.
- Levak-Svajger, B., Svajger, A. and Skreb, N. (1969). Separation of germ layers in presomite rat embryos. *Experientia* **25**, 1311-1312.
- Lumsden, A. and Keynes, R. (1989). Segmental patterns of neuronal development in the chick hindbrain. *Nature* **337**, 424-428.
- Mackenzie, A., Ferguson, M. W. J., Sharpe, P. T. (1991). Hox-7 expression during murine craniofacial development. *Development* **113**, 601-611.
- McKay, R. D. G. (1989). The origins of cellular diversity in the mammalian central nervous system. *Cell* **58**, 815-821.
- Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982). *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory; Cold Spring Harbour, New York.
- Murphy, P., Davidson, D. R. and Hill, R. E. (1989). Segment-specific

- expression of a homeobox-containing gene in the mouse hindbrain. *Nature* **341**, 156-159.
- Murphy, M., Drago, J. and Bartlett, P. F.** (1990). Fibroblast growth factor stimulates the proliferation and differentiation of neural precursor cells *in vitro*. *J. Neurosci Res.* **25**, 463-475.
- Netsky, M. G., and Shuangshoti, S.** (1975). In *The Choroid Plexus in Health and Disease*, (eds., M. G. Netsky and S. Shuangshoti). J. Wright and Sons Ltd., Bristol, England.
- Nieuwkoop, P. D.** (1952). Activation and organization of the central nervous system in amphibia. *J. Exp. Zool.* **120**, 83-108.
- Price, M., Lemaistre, M., Pischetola, M., Di Lauro, R. and Duboule, D.** (1991). A mouse gene related to *Distal-less* shows a restricted expression in the developing forebrain. *Nature* **351**, 748-751.
- Puelles, L., Amat, J. A., and Martinez de la Torre, M.** (1987). Segment-related, mosaic neurogenetic pattern in the forebrain and mesencephalon of early chick embryos: I. Topography of AchE-positive neuroblasts up to stage HH18. *J. Comp. Neurol.* **266**, 247-268.
- Puschel, A. W., Balling, R. and Gruss, P.** (1990). Position-specific activity of the Hox 1. 1 promoter in transgenic mice. *Development* **108**, 435-442.
- Reh, T. A., Nagy, T., and Gretton, H.** (1987). Retinal pigmented epithelial cells induced to transdifferentiate to neurons by laminin. *Nature* **330**, 68-71.
- Schreiber, G., Aldred, A. R., Jaworowski, A., Nilsson, C., Achen, M. G. and Segal, M. B.** (1990). Thyroxine transport from blood to brain via transthyretin synthesis in choroid plexus. *Am. J. Physiol.* **258**, R338-R345.
- Thomas, T., Power, B., Hudson, P., Schreiber, G. and Dziadek, M.** (1988). The expression of transthyretin mRNA in the developing rat brain. *Dev. Biol.* **128**, 415-427.
- Thomas, T., Fletcher, S., Yeoh, G. C. T. and Schreiber, G.** (1989a). The expression of  $\alpha_1$  acid glycoprotein mRNA during rat development. *J. Biol. Chem.* **264**, 5784-5790.
- Thomas, T., Schreiber, G. and Jaworowski, A.** (1989b). Developmental pattern of gene expression of secreted proteins in the brain and choroid plexus. *Dev. Biol.* **134**, 38-47.
- Thomas, T., Stadler, E. and Dziadek, M.** (1992). Effects of the extracellular matrix on fetal choroid plexus epithelial cells: changes in morphology and multicellular organization do not affect gene expression. *Exp. Cell Res.* (in press).
- Toivonen, S. and Saxen, L.** (1967). Morphogenetic interaction of presumptive neural and mesodermal cells mixed in different ratios. *Science* **159**, 539-540.
- Tuckett, F. and Morriss-Kay, G. M.** (1985). The kinetic behaviour of the cranial neural epithelium during neurulation in the rat. *J. Embryol. exp. Morph.* **85**, 111-119.
- Tuckett, F., Lim, L. and Morriss-Kay, G. M.** (1985). The ontogenesis of cranial neuromeres in the rat embryo. I. A scanning electron microscope and kinetic study. *J. Embryol. exp. Morph.* **87**, 215-228.
- Tuggle, C. K., Zakany, J., Cianetti, L., Peschle, C. and Nguyen-Huu, M. C.** (1990). Region-specific enhancers near two mammalian homeobox genes define adjacent rostrocaudal domains in the central nervous system. *Genes Dev.* **4**, 180-189.
- Wilkinson, D. G., Bhatt, S., Cooke, M., Boncinelli, E. and Krumlauf, R.** (1989a). Segmental expression of Hox-2 homeobox-containing genes in the developing mouse hindbrain. *Nature* **341**, 405-409.
- Wilkinson, D. G., Bhatt, S., Chavrier, P., Bravo, R. and Charnay, P.** (1989b). Segment specific expression of a zinc finger gene in the developing nervous system of the mouse. *Nature* **337**, 461-464.
- Yamada, T.** (1990). Regulations in the induction of the organized neural system in amphibian embryos. *Development* **110**, 653-659.
- Yamada, T., Placzek, M., Tanaka, H., Dodd, J. and Jessell, T. M.** (1991). Control of cell patterning in the central nervous system: polarizing activity of the floor plate and notochord. *Cell* **64**, 635-647.

(Accepted 1 October 1992)