Int. J. Dev. Biol. 45: 895-902 (2001)

Technical Article

Mammalian neural tube grafting experiments: an *in vitro* system for mouse experimental embryology

DIEGO ECHEVARRÍA*, CLAUDIA VIEIRA and SALVADOR MARTÍNEZ

Institute of Neuroscience (UMH-CSIC), School of Medicine, Campus de San Juan, Spain

ABSTRACT We have developed a simple experimental technique which consists of explanting the mouse embryo anterior neural tube - the presumptive brain anlage - onto polycarbonate membranes. The neural epithelium of the explants maintained both its original topology and topography for at least two days in culture. Analysis of cell death in the explants by assaying propidium iodide uptake showed high viability of neuroepithelial cells during the culture period. Both the pattern of gene expression and the initial steps of neural cellular differentiation were well preserved, being similar to those which occur in the normal *in vivo* situation. We show here two applications of this tissue culture technique which is similar to that which has been previously employed for avian embryo models. The first involves neuroepithelial grafting experiments by heterotopic transplantation of the *zona limitans intrathalamica* (ZLI) into the mesencephalon and the second consists of fibroblast growth factor (FGF8) protein interaction studies using soaked bead insertions. This *in vitro* system constitutes a powerful experimental embryological tool which can have other applications including time-lapse imaging and electrophysiology.

KEY WORDS: *mouse embryo, tissue transplant, organotypic tissue culture, anterior neural tube, brainstem, vertebrate development, zona limitans*

Introduction

The discovery in recent years of developmental genes which are directly involved in brain patterning and morphogenesis has significantly increased our understanding, at a molecular level, of the regulatory processes which participate in neural development. The study of such genetic regulation in mammals requires new experimental tools to investigate gene function in the early organization of the central nervous system (CNS). The construction of transgenic mice to modify selected gene expression, together with experiments of loss (knockout) and gain (knockin) of function, have partially answered the questions of why and how these genes are important for general embryonic development. Nevertheless, experimental analysis of the molecular and cellular processes which underlie early embryonic regionalization in selected and localized neural domains is currently limited due to the important difficulties involved in the manipulation and accessibility of the mammalian embryo. The development of simple model systems, such as tissue explant culture, can provide a remarkable advance in our understanding of early developmental events, such as local inductive interactions, cell migration and neural connectivity during the patterning and differentiation of various cell types and regions of the CNS.

Most studies concerning neural fate maps and experimental embryology have been performed using the avian embryo, due to its easy accessibility and manipulation *"in ovo"* (Le Douarin, 1973; Martínez *et al.,* 1991; Crossley *et al.,* 1996; Perez-Villegas *et al.,* 1999; Katahira *et al.,* 2000). Several recent studies have reported the use of cultured mouse neural tissue to manipulate parts of the developing brain (Barber *et al.,* 1993; Shimamura and Rubenstein, 1997; Dickinson *et al.,* 1995; Liu *et al.*, 1999; Liu and Joyner, 2001). However, important modifications of neural anatomy due to the *in vitro* conditions make it very difficult to localize the topology of the explanted neural regions. Consequently, an adequate analysis of directional morphogenetic influences acting asymmetrically over neural regions, which normally play a fundamental role during neural development, has not been performed

We present here a simple experimental method which consists of explanting the anterior neural tube - the presumptive brain anlage of

Abbreviations used in this paper: abn, antero basal nuclei; ap, alar plate; bp, basal plate; cf, cephalic flexure; D, Diencephalon; fp, floor plate; gV, trigeminal ganglion; Is, isthmic organizer; M, Mesencephalon; mlf, medial longitudinal fasciculus; os, optic stalk; ov, otic vesicle; R, Rhombencephalon; r1, rhombomere 1; T, Telencephalon; tmesV, mesencephalic tract of the trigeminal nerve; ts, tecto spinal tract; ZLI, zona limitans intrathalamica.

*Address correspondence to: Dr. Diego Echevarría. Institute of Neuroscience (UMH-CSIC). School of Medicine, Campus de San Juan, E-03550 Alicante, Spain. Fax: +34-965-919-555. e-mail: diegoaza@umh.es

0214-6282/2001/$25.00

Printed in Spain
www.ijdb.ehu.es

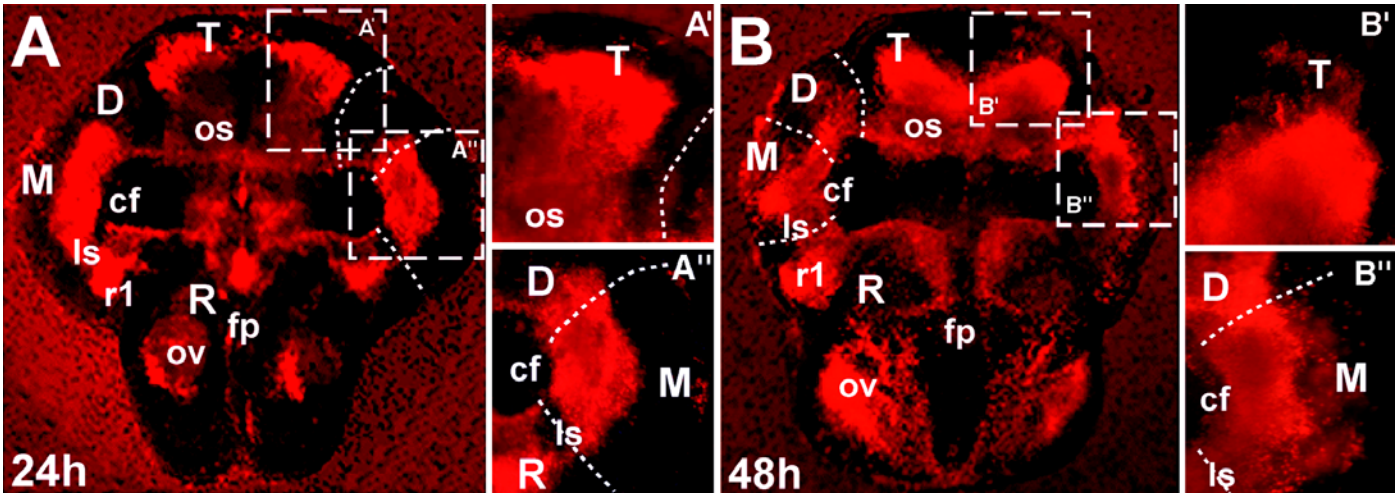

Fig. 1. Viability of tissue explants analyzed by propidium iodide (PI) uptake after (A) 24 and (B) 48 hours of culture. *The red staining at 24 and 48 hours* in vitro *is mainly in the damaged or dead regions of the mesenchyme layer. At 24 hours* in vitro*, the neuroepithelial wall did not incorporate PI* **(A, A′)** *After 48 hours, the number of dead cells in the explant increases in the mesenchyme layer and neuroepithelial wall* **(B′,B′′)***, but both cellular and molecular characteristics are maintained (see also Figs. 2 and 3). The plane of focus of these micrographs was chosen as the superficial neuroepithelial wall in order to visualize PI uptake by the neural cells. Dotted lines indicate the transverse limit domains of the neural tube. A′,A′′,B′and B′′ are higher magnifications of the areas marked by dotted squares in A and B. Abbreviations: cf, cephalic flexure; D, Diencephalon; fp, floor plate; Is, isthmic organizer; M, Mesencephalon; os, optic stalk; ov, otic vesicle; R, Rhombencephalon; r1, rhombomere 1; T, Telencephalon.*

the mouse embryo onto a polycarbonate membrane. Both the original topology and topography of the tissue architecture are well preserved. Using this technique, we have been able to perform tissue grafting and microbead implantation experiments on mammalian embryos. The use of this technique with transgenic mouse lines and mutant mice will facilitate the study of gene function in early neural development. The culture technique reported herein will allow the testing of various experimental hypotheses by grafting genetically modified neuroepithelia into wild type host brain or vice versa, and the possible prolongation of the survival time of the brain anlage in early lethal mutations. In addition, this technique allows visualization of the neuroepithelium (important for electrophysiology and video-microscopy imaging experiments) and a precise assessment of molecular time-dependent changes during early regionalization of the neural tube (Garda *et al.,* 2001).

Results and Discussion

Explant viability

E 9.5 and E 10.5 neural tube explants were cultured for periods of up to 48 hours with a bacteria and yeast contamination rate of less than 1%. Macroscopically, the main brain regions were detectable and tissue integrity appeared to be well preserved after this time in culture. Cell viability was assessed by propidium iodide (PI) uptake after 24 and 48 h in culture (Fig. 1). Two hours after explantation, PI uptake was only seen in those regions subjected to mechanical manipulation during dissection: the dorsal midline, the cephalic flexure and under the otic vesicles (not shown). After 24 hours of culture (Fig. 1A), PI uptake increased in intensity in the cephalic mesenchyme but not at the level of the neuroepithelial wall. Staining was observed mainly in the mesenchyme covering the prosen-

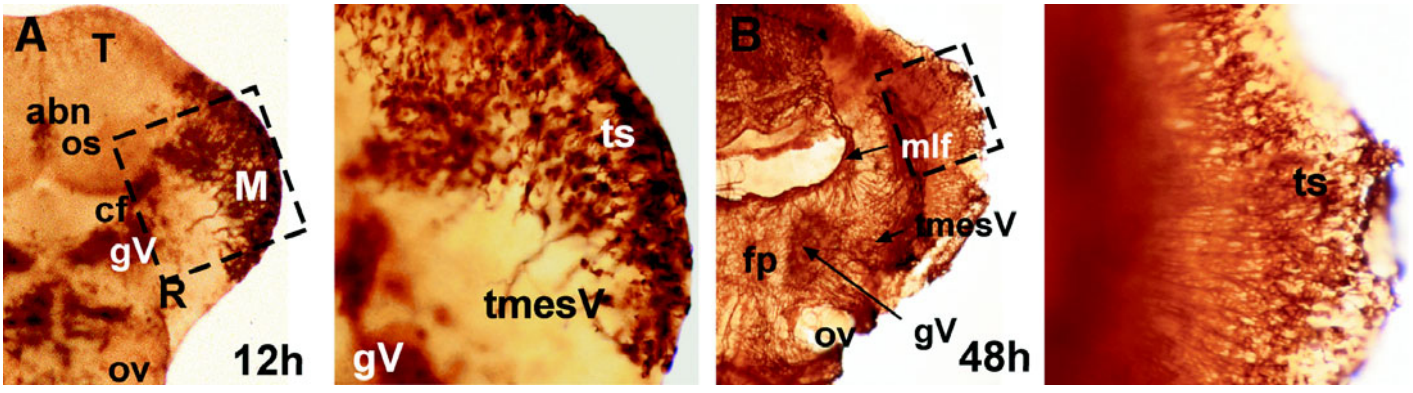

Fig. 2. TuJ-1 immunohistochemistry revealing axonal tract development in cultured mouse neural tube. *After 12 hours of incubation, neuroepithelial cells began to send their projecting axons towards target domains. After 48 hours* in vitro*, the axon projection map is more complex than before but many axonal tracts were still distinguishable. Dashed squares outline the areas shown at higher magnification. Abbreviations: abn, antero basal nuclei; cf, cephalic flexure; gV, trigeminal ganglion; M, Mesencephalon; mlf, medial longitudinal fasciculus; os, optic stalk; ov, otic vesicle; R, Rhombencephalon; T, Telencephalon; tmesV, mesencephalic tract of the trigeminal nerve; ts, tecto spinal tract.*

cephalic and mesencephalic basal plate while dorsal mesenchyme covering the alar plates was not stained. The quantity of PI positive neuroepithelial cells was very low and these were only seen at the level of the basal plate. After 48 hours *in vitro* (Fig. 1B), the number of PI positive cells in the explant increased in the mesenchyme. In the neuroepithelium, more cell death was observed than at 24 hours but the proportion of PI positive cells was less than 20% of the total numbers of cells (data not shown). The neural regions where more cell death was observed included the supraoptic area, the epithalamus, and the basal mesencephalon. The observed pattern of PI positive cells in the neuroepithelium of our explants mimics that associated with naturally occurring, spontaneous cell death, as reported by Hirata and Hall (2000).

Cellular development in the explanted neural tube

We have analyzed the cellular development of some brain structures as well as the pattern of expression of particular genes in different domains of the neural tube during the culture period.

TuJ1 immunostaining

The formation and organization of pioneer axonal tracks *in vivo* has been well described by Easter *et al.* (1993) who used antibodies against the β-tubulin subunit III (TuJ-1) or lipophilic tracers (Mastick and Easter, 1996). We used this immunostaining technique and compared the results (Fig. 2) with those obtained *in vivo* by these authors.

At 12 hours post-explantation (Fig. 2A), the dorsal mesencephalic tract (tmesV) in the mesencephalic alar plate was observed to continue its normal caudoventral course to the area of 5th cranial nerve or trigeminal motor nucleus (gV) in the rostral hindbrain. The antero-basal nuclei, a group of neural cells at the most rostro-ventral midline of the prosencephalon, were also TuJ-1 immunipositive. *In vivo*, these cells are known to prject their axons caudally through the basal plate as the prosencephalic component of the medial longitudinal fasciculus (see below; see also Easter *et al.*1993).

48 hours after explantation (Fig. 2B), axonal patterning was more complex and developed in the explant than that observed at 12 hours. Although the visualization of tracks was more complicated due to the normal development of new projections at different levels of the neural wall (Easter *et al.,* 1993; Mastick and Easter, 1996), some tracts were still detectable. For instance, the tmesV and the mlf are localized in the alar and basal plate of the explanted neural tube respectively (Fig. 2). Orthogonal to these longitudinal fasciculi (tmesV and mlf), transverse dorso-ventral projections are also labeled at different levels of the alar plate. Among them the tecto spinal axons (ts) are clearly visible in the mesencephalic alar plate. Thus, temporo-spatial aspects of initial axon tract formation are conserved during the first 48 h of our organotypic culture, indicating that our explant system can be used to study the initial steps of axon tract development and the regulatory mechanisms involved in this process.

Gene expression patterns

We employed *in situ* hybridization with RNA probes to detect gene transcripts expressed in regions of the neural tube in whole mount embryos and in E 9.5 neural tube explants after 48 hours of culture (Fig. 3). The normal temporo-spatial development of some structures could be observed, including the *zona limitans intrathalamica* (ZLI), a transverse boundary which separates the dorsal and ventral thalamus in the diencephalon (Fig. 3 A,B). ZLI

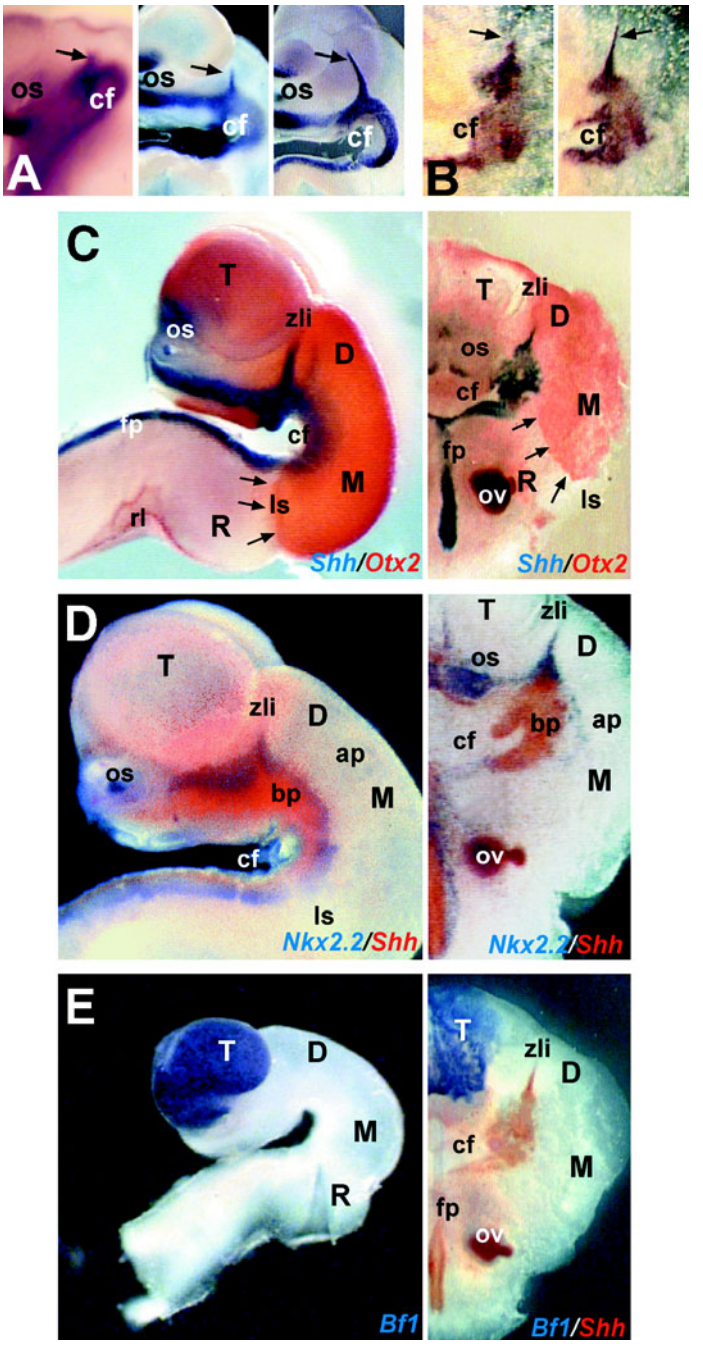

Fig. 3. A range of molecular genetic characteristics are maintained in mouse neural tube tissue culture. Shh *gene expression in the developing* zona limitans intrathalam*ica (A) in vivo and (B) in* vitro*. Arrows indicate the position of the* ZLI *in the diencephalon at different* **(A)***embryological stages (E 9.5, E 10.5 and E 11.5, images from left to right) and* **(B)** *culture periods (24 and 48 h of culture of E 9.5 explants, left and right images respectively).* **(C,D,E)** *Maintenance of localized gene expression patterns in different neural tube domains* in vivo *at E 10.5 and* in vitro *after 48 hours of culture. Note that longitudinal as well as transverse limits are preserved* in vitro*. The arrows indicate the limit of expression of* Otx2 *where the isthmic organizer lies. Abbreviations: ap, alar plate; bp, basal plate; cf, cephalic flexure; D, Diencephalon; fp, floor plate; Is, isthmic organizer; M, Mesencephalon; os, optic stalk; ov, otic vesicle; R, Rhombencephalon; r1, rhombomere 1; T, Telencephalon; ZLI, zona limitans intrathalamica.*

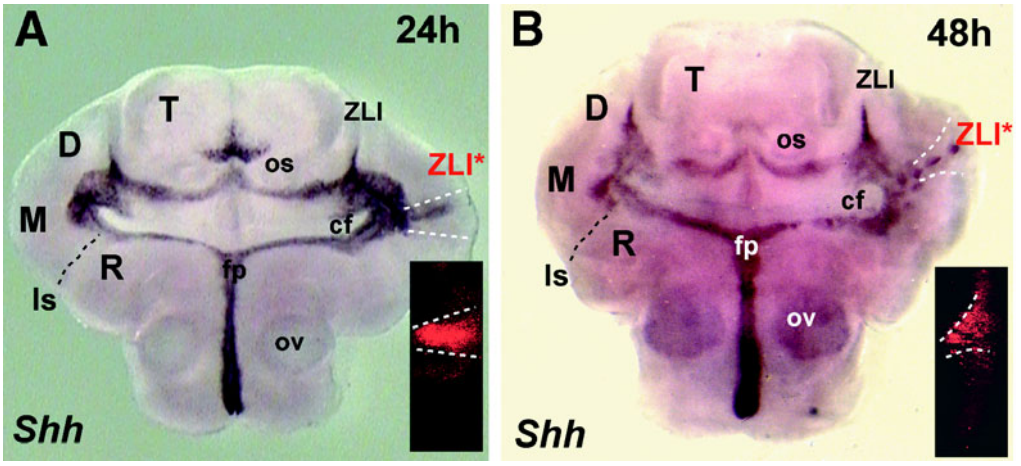

Fig. 4. Ectopic expression of *Shh* in the ZLI tissue grafted into the rostral mesencephalon of E9.5 mouse embryo explants after (A) 24 hours and (B) 48 hours. *The insets at the lower right side of A and B illustrate DiI labeled grafted tissue. White dotted lines indicate the area of the grafted ZLI. ZLI* indicates the ectopic ZLI in the mesencephalon. Black dotted lines mark the mid/hindbrain boundary. Abbreviations: cf, cephalic flexure; D, Diencephalon; fp, floor plate; Is, isthmic organizer; M, Mesencephalon; os, optic stalk; ov, otic vesicle, R, Rhombencephalon; T, Telencephalon; ZLI, zona limitans intrathalamica.*

neuroepithelial cells express the gene *Sonic hedgehog (Shh). In vivo*, at E 9.5 the expression of *Shh* at the ZLI is scarce and short. 24 hours later, it is longer and more extended in the alar plate (Fig. 3A). At E 11.5, the expression of *Shh* is extended along the ZLI spike. A similar expression pattern is also present in the explanted E 9.5 neural tube cultured *in vitro* for 24 h and 48 h (Fig. 3B). These results indicate that the genetic regulation involved in the establishment of the molecular pattern of the ZLI occurs normally during the culture period. Consequently, the initial mechanisms which underlie the formation of localized neural tube areas, such as the ZLI, can be analyzed using our *in vitro* model.

In addition, other transverse boundaries such as the mid-hindbrain boundary can be specifically identified by detecting the pattern of expression of *Otx2* (Fig. 3C; see Garda *et al.,* 2001, Martínez, 2001). Similarly, the tele-diencephalic boundary domain can be identified by detecting the expression of *Bf1* (Fig. 3E; Shimamura and Rubenstein, 1997). Longitudinal domains of the alar, basal and floor plates are also detectable. For instance, longitudinal regionalization can be studied by detecting *Shh* and *Nkx2.2* transcripts. *Shh* is caudally expressed in the floor plate. Subsequently, its expression in the mesencephalon invades the basal plate to follow the basal plate of the diencephalon and secondary prosencephalon (Fig. 3 A,B). The *Nkx2.2* expression pattern dorsally flanks the *Shh* expression domain and, therefore, delimits the boundary between the basal and alar plate through the axial plane of the anterior neural tube (Fig. 3D; Puelles *et al.,* 2001). The normal pattern of *Shh* expression in the ZLI was also found when E 10.5 neural tube embryos were explanted for 48 hours (data not shown).

We have also analyzed the expression pattern of these genes in E 9.5 neural tube explants following longer culture periods. We observed that after 3 days in culture, gene expression patterns change dramatically and ectopically expressing clusters were observed outside their longitudinal and transverse boundaries, suggesting that the organotypic structure had been lost (data not shown). However, we detected that at 60 hours of culture, the normal genetic expression patterns are still present. Therefore, optimal results can be obtained from cultured anterior neural tube maintained for up to 60 hours *in vitro*.

Analysis of the pattern of expression of several developmental genes shows that their normal temporo-spatial patterns of expression are maintained in our *in vitro* culture system, indicating that antero-posterior axis and ventro-dorsal axis formation are adequately maintained in these explants. Many other explant culture techniques have been reported for culturing embryonic brain. Thus, whole embryos can be cultured for at least three to four days *in vitro* (Serbedzija *et al.,*1992; Smits-van Prooije *et al.,*1987, 1988). In these cases, different cell markers or tracers were injected into the yolk sac and endocytosed by all cells surrounding the cavity. Successive analysis of these labeled cells revealed the migration of neural crest cells. With our system we are able to study not only the general migration of cells but also to explore the potentiality of a particular region of the neuroepithelial wall - the neuroepithelial wall is exposed for manipulation - during the first 48 hours within the context of surrounding neuroepithelial regions and external influences. Other *in vitro* techniques have used small pieces of neural tube for explant cultures but in most of the cases, the topology of the tissue is not well defined or missing (Liu *et al.,* 1999, Liu and Joyner, 2001). Our model involves using the anterior neural tube in which morphogenetic activity is preserved along the longitudinal and transverse axes. For instance, the secondary organizers which are active morphogenetic areas, such as the Isthmus (Martínez, 2001), the ZLI (personal observations) and the anterior neural ridge (Ruiz i Altaba, 1998) are present in our explants. This characteristic permits an assessment of the mechanisms that control initial regionalization in the CNS.

Experimental assays on neural tube explants

One of the most interesting aspects of our explant technique is that it permits experimental embryological assays on mammalian embryos. These include grafting tissue experiments and protein interaction studies by means of inserting protein soaked beads into the neuroepithelium, in a same way as has been performed in avian embryo models (Martínez *et al.,* 1991; Teillet *et al.,* 1999; Alvarado-Mallart and Sotelo, 1984).

Graft experiments

Surgical manipulation experiments have been performed using mammalian whole embryos in culture (Wanek *et al.,* 1989). These experiments have involved the amputation or electrocauterization of anterior or posterior limb buds (Naruse *et al.*,1997) but few graft experiments have been performed using the mammalian neural tube (Shimamura and Rubenstein, 1997). In the present work, we

focused our mammalian neural tube graft experiments on the *zona limitans intrathalamica* (ZLI) (Fig. 3 A,B and Fig. 4) in order to have a clear reference structure in our transplant experiments. We assayed whether ectopic ZLI cells can maintain their molecular characteristics. DiI labeled ZLI area was dissected out from the right side of E 9.5 donor mouse embryos (see Experimental Procedures) and ectopically implanted into the right mesencephalon of host explants. These experiments were performed using E 9.5 embryo explants in which the expression of *Shh* at the ZLI is not yet detectable. The explants were analyzed at 24 and 48 hours after grafting (Fig. 4 A,B). Of all the grafts performed in this study (n=23), only 2 did not integrate into the grafted tissue. At 24 hours after grafting (n=11, Fig. 4A), the explanted diencephalic territory was seen to be perfectly integrated into the mesencephalon. Tissue gaps or tissue deformation were not observed, probably due to the intensive proliferative activity that embryos have during early embryogenesis. *In situ* hybridization to the *Shh* gene (a marker for the ZLI) revealed an ectopic *Shh* signal from the basal plate to the alar plate, mimicking in the mesencephalon the normal expression of *Shh* in the ZLI. In many cases, as shown in Fig. 4A, the continuity of *Shh* expression from the basal plate could be observed. This data indicates that the grafted area maintains the molecular genetic properties and integrity of the original ZLI under heterotopic influences. At 48 hours of grafting (Fig. 4B), the ectopic *Shh* positive signal was still observed in the grafted tissue (n=10). However, the morphology of the grafted tissue changed. The grafted region is narrower than that observed at 24 hours and therefore more visually detectable than before. These morphological changes may be due to a differential growth time scale of the host neighbor tissue in relation to the donor tissue. The expression of *Shh* was still present in the donor tissue in the form of an ectopic transverse line, which was sometimes discontinuous (Fig. 4B). These results demonstrate that the ZLI neuroepithelial anlage maintains *Shh* expression when ectopically placed in the alar mesencephalon, suggesting that the ZLI is committed to expressing this transcript at E 9.5 before expression is detectable. Thus, grafted tissue is preserved and integrated perfectly in the host and it maintains certain native molecular characteristics even after 48 hours of culture. This approach can be used for the study of cell movements in the neuroepithelial wall (with GFP neuroepithelial cells or tissue) as well as for identifying molecular properties in relation to embryo topology in mammals (morphogenetic inductors; see Martínez, 2001). Manipulation of selected neuroepithelial regions for homotopic and heterotopic tissue grafts is not possible in other culture system (whole culture embryo) since the neural tube in those cases is not accessible for experimentation.

Molecular interaction experiments

Our explant culture system can also be used to analyze the molecular and cellular effects of ectopic expression of molecular factors with morphogenetic activity, by means of implanting microbeads. We chose the molecule FGF8, a key molecule in the formation of the isthmic organizer in the developing neural plate (Garda *et* al., 2001), to analyze whether the molecular interactions underlying FGF8 induction in chicks are conserved in mouse embryos (Crossley *et al.,* 1996; Martínez *et al., 1999*). We inserted FGF8 soaked beads (R&D) into the neuroepithelial layer of the mesencephalic region of explants from E9.5 and E10.5 mouse embryos (Garda *et* al., 2001).

In E9.5 explants, we observed that after 24 hours in culture with FGF8-bead insertion, the neuroepithelium around the bead was induced to express *Gbx2* (5 of 6 cases Fig. 5A). The level of *Gbx2* expression was higher in the ectopic domain than in the normal isthmic region and extended more to the dorsal side of the bead than to its ventral side (Fig. 5 A,C). Analysis of *Otx2* transcripts in 10 explants after 24 hours in culture showed that this gene was expressed in the cells which touched the bead and therefore, co-expressed *Gbx2* (data not shown). 48 hours after culture, the induced expression of *Gbx2* in the cells around the bead was clear, but now a domain of *Otx2* repression was observed (Fig. 5 B,C). This zone was more extensive dorsally than ventrally and at this larger dorsal level, a negative domain of *Gbx2* expression was also observed between the *Otx2* repression border and the *Gbx2* induction domain around the bead (Fig. 5C). In explants from E 9.5 embryos, we observed similar inductive results in 18 out of 22 experiments, but in explants from E 10.5 mouse embryos, no effects were ever observed under the present conditions (n=10, data not shown). This suggests that the potential of the mesencephalic epithelium to express *Gbx2*

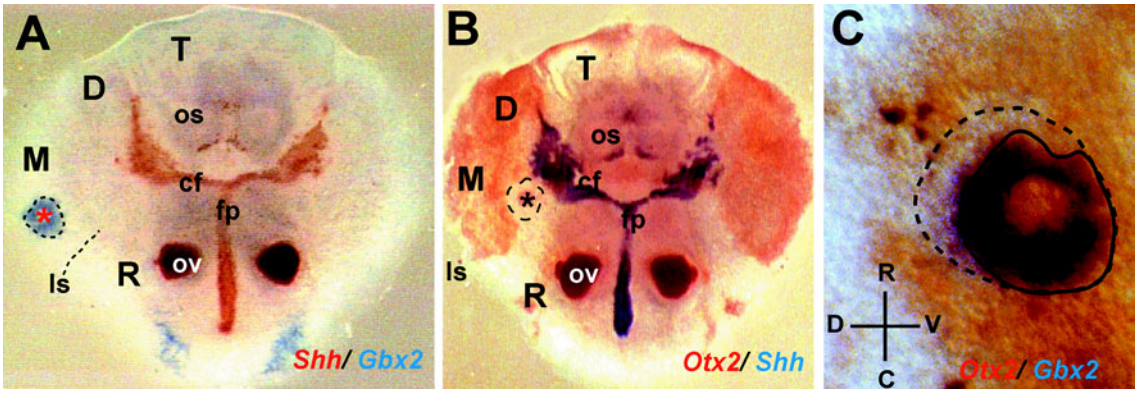

Fig. 5. Molecular effects of ectopically applied FGF8, delivered by implantation of FGF8-impregnated beads into the mesencephalon, after 24 and 48 hours of culture. **(A)** *Ectopic induction of the Gbx2 transcript after implantation of an FGF8-soaked bead in the mesencephalon.* **(B)** *Ectopic repression of the Otx2 gene after implantation of an FGF8-soaked bead in the mesencephalon. Black dashed circles outline the areas of inductive/repressive activity of ectopically applied FGF8. Asterisks indicate the positions of the beads.* **(C)** *A closer view of the molecular effects of the FGF8 beads on the mesencephalon. The dashed circle outlines the area in which the repressive effect of FGF8 on* Otx2 *expression can be observed. The black circle indicates the area where* Gbx2 *is induced due to the presence of the FGF8 bead. Abbreviations: cf, cephalic flexure; D, Diencephalon; fp, floor plate; Is, isthmic organizer; M, Mesencephalon; os, optic stalk; ov, otic vesicle, R, Rhombencephalon; T, Telencephalon; ZLI, zona limitans intrathalamica.*

in response to FGF8 disappears at this stage. The results demonstrate the higher level of efficiency and sensitivity of this method to analyze molecular interactions in the neuroepithelium, since bead insertion in cultured whole embryos is much less precise.

Experimental Procedures

Animal experimentation in this study was carried out in strict accordance with the relevant national and international laws and policies (EEC Council Directive 86/609, OJ L358. 1, Dec. 12, 1987; NIH *Guide for the care and use of laboratory animals*. NIH publication 85-23, 1985; and the Society for Neuroscience, Jan. 1995).

Neural tube dissection and organ tissue culture

Timed pregnant ICR mice were killed by cervical dislocation and embryos were dissected from decidual tissue in chilled Hibernate solution (Gibco-Life technologies). The day of observation of a vaginal plug was defined as E 0.5 and this definition was corroborated after extraction of embryos by comparing morphological structures with those of the atlas of mouse development (Kaufman, 1999).

Heads from E 9.5 and 10.5 embryos were cut at the level of rhombomeres (r) r4/ r5 and the otic vesicle (ov) was taken as a caudal reference. Thereafter, the neural tube was opened along the dorsal midline (roof plate), by cutting open the tube in a caudal to rostral direction, to the level of the *lamina terminalis*. In order to flatten the tissue, the floor plate at the level of the cephalic flexure (cf) was also cut with the help of microtip forceps. Thus, the neural tube was maintained like an "open book". No mesenchyme was removed by enzymatic digestion. Explant tissue was transferred to sterile Petri dishes and placed (ventricular part facing up) on floating polycarbonate membrane filters of 8 μm pore size (Nunc) with 10% fetal bovine serum in DMEM culture medium (Gibco-Life Technologies). Glutamax (2mM; Gibco-Life Technologies) and Penicillin-Streptomycin (100U/ml-100 μg/ml; Gibco-Life Technologies) were added to the culture medium. Explants were generally maintained for up to 48 hours in an incubator at 37°C, with 5% CO_2 and 95% humidity, although some experiments lasted for up to 60 and 72 hours in culture in order to analyze the maintenance of molecular characteristics (see Results and Discussion). All procedures were carried out under sterile conditions.

Experimental manipulation of the organ tissue culture

One of the advantages of this *in vitro* technique is that the explanted neural tissue behaves as the original tissue does *in vivo*, in the sense that the organotypical structure of the tissue is preserved. In addition, the neural epithelium can be easily manipulated due to its exposure of the ventricular side facing up. Thus, some of the embryological issues regarding early development of the CNS can be explored with relative ease.

Grafting experiments

Grafting experiments were carried out using donor and host embryos from the same litter. Before dissection, opened neural tubes of donor embryos were incubated with the lipophilic tracer DiI (100 ng/ml final concentration) (Honing and Hume, 1989). DiI was diluted in 100% ethanol (1 mg/ml; DiI-E) and this solution was re-diluted 1:100 in 0.3 M glucose (DiI-E-S). Finally DiI-E-S was diluted 1:100 in culture medium at 37°C. The donor embryos were incubated in this culture medium containing DiI-E-S for one hour. After incubation, tissue was washed briefly in culture medium and prepared for microscopic dissection of the *zona limitans intrathalamica* (ZLI) neuroepithelium. Tissue grafting was performed using either fine tungsten needles or by sucking the relevant portion of tissue using glass capillaries of different tip size diameters. The presumptive ZLI region of E 9.5 mice was dissected and isolated from the donor, transferred to culture medium by a glass pipette, and ectopically inserted in the host mesencephalon into a previously made equivalent hole (heterotopic grafts). Visualization of the ZLI was carried out by detecting the expression of sonic hedgehog (*Shh)* as a marker of the ZLI by *in situ* hybridization techniques (see below). The labeled and grafted tissue were visualized using an upright photomicroscope (Leica) equipped with the corresponding excitation filter.

Implantation of protein-soaked beads

We have analyzed the consequences of heterochronic (different time/stage) and heterotopic (different region) induction of an isthmic organizer by implantation of microbeads soaked in the b isoform of fibroblast growth factor 8 (referred to as FGF8) into the neural tube of explant cultures (Garda *et al.,* 2001). Heparin acrylic beads (Sigma) were rinsed in PBS 4 to 6 times and then soaked in 5 μl FGF8 solution (1 mg/ml; R&D), for 1 hour at 4°C. The beads were then rinsed three times in PBS (phosphate buffer saline 0.1 M) and thereafter implanted in the neural tube. Control beads were soaked only in PBS and implanted in the same manner. After 24 to 48 hours of culture, explants were fixed in 4% formaldehyde in PBS, freshly prepared from paraformaldehyde and processed for *in situ* hybridization (see below). Structural development and variations in gene expression in the experimental samples were analyzed using the contralateral side of the neural tube as a control, as well as the parallel detection of the same genes in normal isochronic mouse embryos. In all cases, the control side of the experimental samples showed gene expression patterns which were similar to those observed in normal mouse embryos (see Results and Discussion).

Histochemistry

Viability assays

The presence of dead or dying cell was assessed in explant cultures and control embryos using the fluorescent dye propidium iodide (PI; Sigma). PI enters cells when the membrane is damaged, and upon binding to exposed DNA, it becomes highly fluorescent. Explants and control embryos were incubated for two hours with PI (25 μg/ml; Sigma) in culture medium, according to Hsu *et al.* (1994). Thereafter, they were rinsed twice in culture medium, and visualized using the corresponding wavelength for rhodamine under an upright fluorescence photomicroscope (Leica). Qualitative analysis of PI uptake was performed after 2, 24 and 48 hours of explantation as well as at the corresponding control stages.

Immunohistochemistry

Explant cultures and control embryos were fixed overnight at 4ºC in PBS containing 4% formaldehyde, freshly prepared from paraformaldehyde. After fixation, the samples were removed from the membrane and permeabilized according to Easter *et al.* (1993). Briefly, explants were dehydrated in an ascending series of ethanol and cleared in xylene. Then, they were incubated in acetone at -20ºC for 10 min and rehydrated.

Non-specific reactions were blocked by incubation in PBS containing 0.2% gelatin, 0.1% sodium azide, 0.25% Triton X-100 and 0.1 M lysine (blocking solution) for 60 min. After rinsing, explants were incubated overnight with an antibody to a neuron-specific class III β-tubulin (TuJ-1, 1:500 in blocking solution, CRP inc.). Subsequently, they were washed in PBS and incubated in biotinylated goat anti-mouse IgGs (1:200 in blocking solution), rinsed again in PBS, and incubated for an hour in peroxidase-conjugated streptavidin (ABC Elite kit; Vector Laboratories) in PBS. The developing reaction was carried out with 0.05% 3,3'-diaminobenzidine and 0.01% hydrogen peroxide in 50 mM Tris-HCl pH 7.6. Afterwards, explants were dehydrated to absolute alcohol, cleared in xylene, coveredslipped in Eukitt and photographed using a dissecting microscope (Leica).

In situ *hybridization analysis*

Explant tissue cultures and the equivalent control embryos were fixed in 4% formaldehyde at 4ºC overnight, washed in PBS and dehydrated through ascending methanol stored at -20°C before being processed for *in situ* hybridization as described by Shimamura *et al.* (1995). Antisense riboprobes were prepared from plasmids kindly provided by A. McMahon *(Shh)* G. Martin *(Fgf8),* A. Simeone *(Otx2 and Bf1)* and K. Shimamura *(NKx2.2)*. For two color staining, NBT/BCIP (blue) and INT/BCIP (red) (Boehringer/Mannheim) were used as chromogenic substrates for alkaline phosphatase. After hybridization, embryos and explants were washed in PBT (0.1% Tween 20 in PBS) and refixed in 4% formaldehyde overnight at 4ºC and stored in 70% glycerol in PBS at 4°C with 0.1% sodium azide. Embryos as well as explants were analyzed and photographed under a dissecting microscope (Leica).

Conclusions

In the present study, we have described a simple *in vitro* method for the experimental analysis of early development of the mammalian neural tube. The method involves explanting the opened neural tube with the ventricular side facing up, making the neuroepithelial wall more accessible to manipulation. In this system, neural topography and cellular morphology is maintained for 48 hours in culture, and reproduces in time and space the initial molecular and cellular processes normally observed *in vivo*. After 60 hours in culture, the tissue changes morphologically and molecularly suggesting that the organotypic structure is no longer preserved. This culture technique offers a new approach for developing original experimental hypotheses, by grafting genetically modified neuroepithelium into wild type host brain or vice versa, and for prolonging the survival time of the brain anlage in early lethal mutations. Moreover, this *in vitro* model permits experimental studies which require accessibility and visualization of the neuroepithelium, such as electrophysiological studies and time-lapse imaging studies.

Acknowledgements:

We would like to thank Dr. Diego. Sánchez for useful comments on the manuscript and M. Ródenas and F. Almagro for technical assistance. The authors also wish to thank the agency ACTS (acts@euskalnet.net) for having corrected the English version of our paper. This work was supported by postdoctoral fellowship to D.E. from the Basque Government (BIF00.80.DE) and the European Union (UE QLRT-1999-31556 and UE QLRT-1999).

References

ALVARADO-MALLART, R.M. and SOTELO, C. (1984). Homotopic and heterotopic transplantations of quail tectal primordia in chick embryos: organization of the retinotectal projections in the chimeric embryos. *Dev. Biol.* 103: 378-398.

BARBER, R.P., PHELPS, P.E. and VAUGHN, J.E. (1993). Preganglionic autonomic motor neurons display normal translocation patterns in slice cultures of embryonic rat spinal cord. *J. Neurosci.* 13: 4896-4907.

CROSSLEY, P.H., MARTINEZ, S., and MARTIN, G.M. (1996). Midbrain development induced by FGF8 in the chick embryo. *Nature* 380:66-68.

DICKINSON, M.E., SELLECK, M.A., MCMAHON, A.P. and BRONNER-FRASER M. (1995). Dorsalization of the neural tube by non-neural ectoderm. *Development* 121: 2099-2106.

EASTER, S.S.JR., ROSS, L.S., and FRANKFURTER, A. (1993). Initial tract formation in the mouse brain. *J. Neurosci.* 13:285-299.

GARDA, A.-L., ECHEVARRIA, D., and MARTINEZ, S. (2001). Neuroepithelial coexpression of Gbx2 and Otx2 precedes Fgf8 expression in the isthmic organizer. *Mech. Dev.* 101:111-118.

HIRATA, M. and HALL, B.K. (2000). Temporospatial patterns of apoptosis in chick embryos during the morphogenetic period of development. *Int. J. Dev. Biol.* 44: 757-768.

HONNIG, M.G., and HUME, R.I. (1989) DiI and DiO: versatile fluorescent dyes for neural labelling and pathway tracing. *Trends Neurosci.* 12: 333-341

HSU, S.S., NAVEL, D.W, TUCKER, A., MALOUF, A.T. and WINN, H.R. (1994). Adenosinergic modulation of CA1 neuronal tolerance to glucose deprivation in organotypic hippocampal cultures. *Neurosci. Lett.* 178: 189-192.

KATAHIRA, T., SATO, T. SUGIYAMA, S. OKAFUJI, T., FUNAHASHI, J. and NAKAMURA, H. (2000). Interaction between Otx2 and Gbx2 defines the organizing center for the optic tectum. *Mech. Dev.* 91:43-52.

KAUFMAN, M.H. (Ed.) (1999) *Atlas of mouse development*. Academic Press, London.

LE DOUARIN, N.M. (1973) A biological cell labelling technique and its use in experimental embryology. *Dev. Biol.* 30: 217-222.

LIU, A., LOSOS, K., and JOYNER, A.L. (1999). Fgf8 can activate Gbx2 and transform regions of the rostral mouse brain into a hindbrain fate. *Development* 126: 4827-4838.

LIU, A., and JOYNER, A.L. (2001) En and Gbx2 play essential roles downstream of FGF8 in patterning the mouse mid/hindbrain region. *Development* 128: 181-191.

MARTINEZ, S. (2001) The isthmic organizer and the brain regionalization *Int. J. Dev. Biol.* 45: 367-371.

MARTÍNEZ, S.; CROSSLEY, P.H.; COBOS, I.; RUBENSTEIN, J.L.R and MARTIN, G.R. (1999). FGF-8 induces an isthmic organizer and isthmocerebellar development in the caudal forebrain via a repressive effect on Otx2 expression. *Development* 126: 1189-1200.

MARTINEZ, S., WASSEF, M., and ALVARADO-MALLART, R.M. (1991). Induction of mesencephalic phenotype in the 2 day old chick prosencephalon is preceded by the early expression of the homeobox gene En. *Neuron* 6: 971-981.

MASTICK, G.S and EASTER, S.S.JR. (1996). Initial organization of neurons and tracts in the embryonic mouse fore- and midbrain. *Dev. Biol. 173:* 79-94.

NARUSE, I., KEINO, H. and TANIGUCHI, M. (1997) Surgical manipulation of mammalian embryos *in vitro*. *Int. J. Dev. Biol.* 41: 195-198.

PEREZ-VILLEGAS, E.M., OLIVIER, C., SPASSKY, N., PONCET, C., COCHARD, P., ZALC, B., THOMAS, J.-L., and MARTINEZ, S. (1999) Early specification of oligodendrocytes in the chick embryonic brain. *Dev. Biol.* 216:98-113.

PUELLES, L., KUWANA, E., PUELLES, E., BULFONE, A., SHIMAMURA, K., KELEHER, J., SMIGA, S., and RUBENSTEIN J.L. (2000). Pallial and subpallial derivatives in the embryonic chick and mouse telencephalon, traced by expression of genes Dlx2, Emx1, Nkx2.1, Pax6 and Tbr1. *J. Comp. Neurol.* 424: 409-438.

RUIZ I ALTABA, A. (1998) Deconstructing the organizer. *Nature* 391:348-349

SERBEDZIJA, G.N., BRONNER-FRASER, M. and FRASER, S. (1992). Vital dye analysis of cranial neural crest cell migration in the mouse embryo. *Development* 116: 297-307.

SHIMAMURA, K., HARTIGAN, D.J., MARTINEZ, S., PUELLES, L. and RUBENSTEIN, J.L. (1995). Longitudinal organization of the anterior neural plate and neural tube. *Development* 121: 3923-3933.

SHIMAMURA, K. and RUBENSTEIN, J.L. (1997). Inductive interactions direct early regionalization of the mouse brain. *Development* 124: 2709-2718.

SMITS-VAN PROOIJE, VERMEIJ-KEERS, CHR., DUBBELDAM, J.A. MENTINK, M.M.T., and POELMANN, R.E., (1987). The formation of mesoderm and mesectoderm in presomite rat embryos cultured *in vitro*, using WGA-Au as a marker. *Anat. Embryol.* 176: 71-77

SMITS-VAN PROOIJE, VERMEIJ-KEERS, CHR., POELMANN, R.E., MENTINK, M.M.T., and DUBBELDAM, J.A. (1988). The formation of mesoderm and mesectoderm in 5- to 41-somite rat embryos cultured *in vitro*, using WGA-Au as a marker. *Anat. Embryol.* 17: 245-256

TEILLET, M.A., ZILLER, C. and LE DOUARIN, N.M. (1999). Quail-chick chimeras. *Methods Mol. Biol.* 97:305-18.

WANEK, N., MUNEOKA, K. and BRYANT, S.V. (1989). Evidence for regulation following amputation and tissue grafting in the developing mouse limb. *J. Exp. Zool.* 249: 55-61

Received: August 2001
Reviewed by Referees: September 2001
Modified by Authors and Accepted for Publication: November 2001