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A new method of embryonic culture for assessing global changes in brain organization

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Abstract

While dissociated, reaggregated cells and organotypic slice cultures are useful models for understanding brain development, they only partially mimic the processes and organization that exist *in vivo*. Towards bridging the gap between *in vitro* and *in vivo* paradigms, a method for culturing intact brain tissue was developed using whole cerebral cortical hemispheres in which the anatomical and cellular organization of nervous system tissue is preserved. Single, free-floating telencephalic hemispheres were dissected from embryonic mice and placed into defined culture medium on an orbital shaker. Orbital shaking was necessary for optimal growth, and cortices grown under these conditions closely approximated *in vivo* parameters of cell division, differentiation, migration and cell death for up to 24 h. In addition to wild-type cultures, the method was compatible with genetically altered tissues. One particular advantage of this method is its ability to reveal global anatomical alterations in the embryonic brain following exposure to soluble growth factors. This method should thus be helpful for assessing a wide range of soluble molecules for their systemic effects on the embryonic brain.

Keywords: Development; Embryonic; Cerebral cortex; *Ex vivo*; Cell culture

1. Introduction

The size and shape of the cerebral cortex are determined during neurogenesis, when cells undergo proliferation, differentiation, migration and cell death in the developing brain (Caviness et al., 1995; Haydar et al., 1999a,b; Pompeiano et al., 2000). These various neurogenic processes depend on local cellular interactions that are altered by the disruption of an intact central nervous system (Bittman et al., 1997; Linden et al., 1999; Murciano et al., 2002).

Dissociated, reaggregated embryonic brain cells and organotypic slice cultures are simple models for understanding cerebral cortical development (Berglund et al., 2004; Ghosh et al., 1994; Haydar et al., 1999a,b). While these systems allow for precise control of culture conditions, they do not retain the spatial organization of the brain, and consequently, they only partially mimic the underlying neurogenic processes that occur *in vivo*. Here, the potential use of cultured whole cerebral cortex hemispheres was explored for studying mechanisms dependent on the anatomical organization of the nervous system. This model takes advantage of the controlled environment of *in vitro* systems while preserving the integrity of cerebral cortical tissue.

In 1961, Moscona described the use of gyratory rotation for culturing suspensions of embryonic cells (Moscona, 1961). While this orbital shaking was necessary to promote the aggregation and formation of three-dimensional spheres from the cell suspensions, it also improved the aeration and diffusion of nutrients to the cells. To promote the aeration and diffusion of nutrients while culturing whole brain hemispheres, orbital shaking was explored here.

The present model also permits the study of soluble factors for their possible effects on cortical architecture. Extrinsic signaling molecules such as neurotrophins, fibroblast growth factor 2 (FGF2), epidermal growth factor, pituitary adenylyl cyclase activating peptide, and insulin-like growth factor 1 are known

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to alter cell cycle, migration, differentiation and cell death in neuronal cells (Barde, 1994; Bartlett et al., 1994; Bondy and Cheng, 2004; Dicicco-Bloom et al., 1998; Farinas et al., 2002; Ferguson and Slack, 2003; Ford-Perriss et al., 2001; Fukushima et al., 2002; Ghosh and Greenberg, 1995; Kingsbury et al., 2003; Meirer et al., 2001; Ostenfeld and Svendsen, 2004; Temple and Qian, 1995; Vaccarino et al., 1999; Waschek, 2002; Wong and Guillaud, 2004); however, the roles these molecules play in shaping cerebral cortical morphology are poorly understood.

Using orbital shaking in defined media, morphology and neurogenic processes in whole cerebral hemispheres were assessed. The novelties of this free-floating preparation are that it approximates *in vivo* organization while preserving an intact cortical hemisphere and permits exposure of intact brain tissue to a wide range of soluble factors that can be delivered under controlled conditions.

2. Material and methods

2.1. Animals

Animal protocols were approved by the Animal Subjects Committee at The Scripps Research Institute and conform to National Institutes of Health guidelines and public law. Timed-pregnant BALB/c or C57Bl/6 females were anesthetized by halothane inhalation and sacrificed by cervical dislocation.

2.2. Dissection of cortical hemispheres

Embryos were removed at embryonic day 14 (E14) and placed into a 100 mm × 15 mm tissue culture (TC) dish containing serum-free medium consisting of Opti-MEM I (Gibco-BLR, Cat. #31985-070) with 20 mM glucose, 55 mM 2-mercaptoethanol and 1% penicillin/streptomycin. Brains of embryos were then dissected in individual 60 mm × 15 mm dishes containing serum-free medium. Specifically, an anterior horizontal cut above the eyes and a posterior horizontal cut through the brainstem were performed using fine forceps (#5, Fine Science Tools). Starting at the lateral edge, skull tissue was gently removed using forceps to free the entire brain from the head of the embryo (Fig. 1A). The two cerebral cortical hemispheres were separated along the midline (white dashed line, Fig. 1B) and cut away from the remaining brain using a No. 11 scalpel blade (black dashed line, Fig. 1B). Excess midbrain tissue along the midline of each hemisphere was removed to expose the lateral ventricles for ample diffusion of medium and nutrients (black arrow, Fig. 1C). The meninges were kept intact. This procedure takes approximately 30 min per pregnant female (approximately 6–9 embryos) to be completed.

2.3. Ex vivo cultures

Using a P1000 Pipetman and a cut pipette tip, each cortical hemisphere was transferred with 1 ml of medium from the 60 mm × 15 mm dish to an individual well of a 12-well TC plate containing 1 ml of fresh medium (total volume within each individual well was 2 ml). TC plates were then placed on a shaker

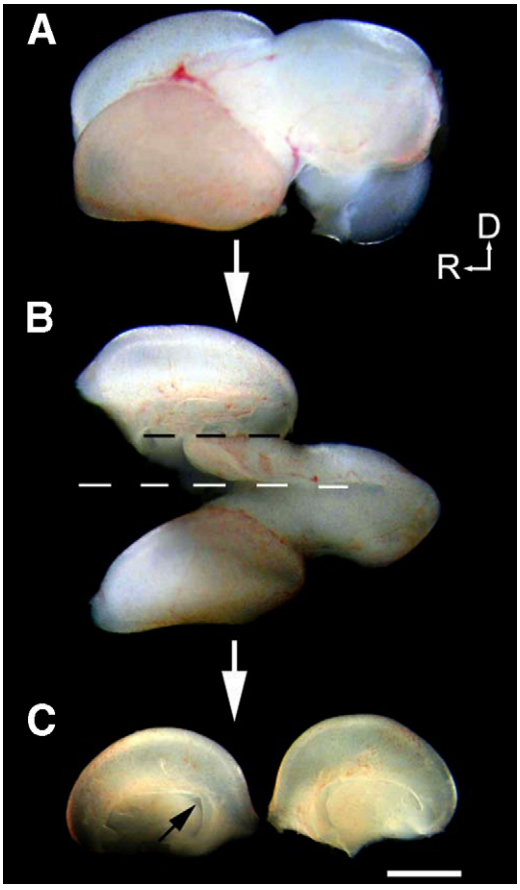

Fig. 1. Dissection of cortical hemispheres. (A) E14 brains were removed from the skull. (B) Cortical hemispheres were divided along the midline (white dashed line). (C) Hemispheres were dissected away from the rest of the brain (black dashed line) and the lateral ventricles were exposed (black arrow) to allow adequate diffusion of nutrients. The meninges were left intact. D: dorsal; R: rostral. Scale bar, 1 mm.

table inside a tissue culture incubator. Cortices were cultured for 24–48 h at 37 °C in 5% CO_2 with mild agitation (approximately 70 rpm), similar to previous descriptions (Rehen et al., 1996).

For growth factor experiments, hemispheres were cultured in medium containing 1 μM lysophosphatidic acid (LPA; Oleoyl-LPA; Avanti Polar Lipids, Alabaster, AL) in 0.1% fatty-acid free bovine serum albumin (FAFBSA; Sigma) to serve as a carrier for LPA, or 40 ng/ml of FGF2 (R&D Systems, Minneapolis, MN) while the opposite hemisphere was cultured in control medium containing 0.1% FAFBSA or serum-free medium alone (Fig. 2).

2.4. Preparation of growth factors

For LPA, 220 μl sterile water was added to 1 mg LPA powder to make a 10 mM solution. This 10 mM solution was then diluted with 10% FAFBSA to make a 100 μM solution. Either 20 μl of 100 μM LPA in 10% FAFBSA or 20 μl of 10% FAFBSA was added to each well containing a cortical hemisphere in 2 ml final volume of defined medium. The final concentration of LPA per well is 1 μM in 0.1% FAFBSA. For FGF, FGF powder is

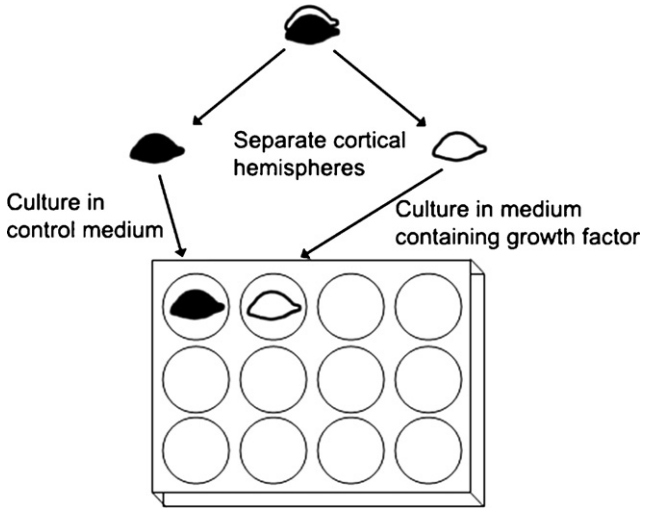

Fig. 2. Method used for assessing the effects of extrinsic factors on cortical anatomy. Cortical hemispheres from the same animal were separated along the midline and one hemisphere was cultured in medium containing a growth factor while the other hemisphere was cultured in control medium.

rehydrated to 20 ng/μl in 0.1% BSA. We then add 4 μl of this solution to a cortical hemisphere in 2 ml total medium.

2.5. Tissue processing

At the end of culture, hemispheres were fixed overnight at 4 °C by adding 2 ml of 8% paraformaldehyde (para) in 0.1 M phosphate buffered saline (PBS; pH 7.4) to the 2 ml of medium within each TC well. After fixation, cortices were cryoprotected by replacing the fixative with a 15% sucrose solution in PBS for 30 min, followed by a 30% sucrose solution overnight at 4 °C. Cortices were then embedded in 22 mm × 22 mm × 20 mm disposable embedding molds (Polyscience, Inc., Warrington, PA) using Tissue-Tek embedding matrix (Sakura, Torrance, CA). Cortices were oriented at the bottom of the mold in the sagittal plane such that the lateral ventricles were visible from the dorsal surface (see orientation in Fig. 1C). Brain molds were rapidly frozen on dry ice and transferred to −20 °C until sectioning. For *in situ* end-labeling plus (ISEL$^+$), cultured cortices were immediately fresh frozen, rather than para fixed, and transferred to −20 °C until sectioning. Both fixed and unfixed tissue was cut at 10 μm on a cryostat, mounted onto Superfrost Plus slides (Fisher Scientific) and air-dried.

Freshly isolated cerebral cortices or in some cases, whole embryos, were immediately frozen in Tissue-Tek and sectioned as described above for use as *in vivo* controls.

2.6. Immunolabeling and ISEL$^+$ labeling

Monoclonal antibodies and rabbit polyclonal antisera used for staining progenitor cells and postmitotic neurons included α-Ki67 (1:100, Pharmingen), α-neuronal class III β tubulin (1:500, Chemicon), α-phospho-histone H3 (α-phospho-H3, 1:500, Upstate Biotechnology) and α-cleaved caspase-3 (1:50, Cell Signaling). Primary antibodies were detected with cy3- or FITC-conjugated goat α-mouse (1:200) or cy3-conjugated donkey α-rabbit (1:500) antibodies (Jackson Immunoresearch). Tissue was first blocked with 2.5% bovine serum albumin and 0.3% Triton X-100 in PBS for 1 h. Tissue was then rinsed in PBS three times for 5 min each and incubated in the primary antibody/antiserum overnight. Sections were rinsed in PBS three times for 10 min each and incubated in secondary antibody for 1 h. Tissue was rinsed again in PBS three times for 5 min each with the first wash containing the nuclear counterstain, 4′,6′-diamino-2-phenylindole (DAPI; Sigma). Sections were coverslipped with Vectashield (Vector Laboratories).

ISEL$^+$ labeling was performed as previously described (Blaschke et al., 1996; Blaschke et al., 1998) using digoxigenin-11-dUTP and alkaline phosphatase-conjugated anti-digoxigenin Fab fragments. Alkaline phosphatase activity was detected using 4-nitroblue tetrazolium chloride and X-phosphate to yield a black reaction product. After the reaction, sections were stained with DAPI, washed in Milli-Q H_2O and coverslipped with Crystal/Mount.

2.7. Quantification of labeled cells

Sagittal sections through the center of the lateral ventricle were used for analysis. For quantification of immunolabeled cells, two cross-sections (∼200 μm across by width of cortex from pial to ventricular surface) from anterior, middle and/or posterior cortex that were matched for location in *ex vivo* and *in vivo* hemispheres or in control and treated hemispheres were analyzed in six animals for each treatment group ($N = 12$ total sections per group). For ISEL$^+$ labeled cells, three cross-sections matched for location in *ex vivo* and *in vivo* hemispheres were analyzed in three animals from each treatment group ($N = 9$ total sections per group). All sections were captured with a Zeiss AxioCam digital camera attached to a Zeiss Axioscope using either a X20 dry objective lens or a X25 oil objective lens. Labeled cells were scored in Adobe Photoshop 6.0 (Adobe Systems) and quantified in NIH image 1.62 (NIH). Experimenters were blind to the experimental conditions during counting. For mitotic analyses of phospho-H3-labeled cells, counts were expressed as a percentage of total cell number per cross-section, determined by counting counterstained DAPI nuclei. Comparisons between experimental groups were made using paired and unpaired *t*-tests in Statview 5.0 (SAS Institute Inc.). Images were prepared in Photoshop 6.0.

3. Results

3.1. Brain morphology is preserved in ex vivo culture

With orbital shaking, cortical hemispheres displayed a healthy anatomical appearance after 24 h in culture (Fig. 3B), maintaining the morphology observed *in vivo* (i.e. a freshly isolated cortex; Fig. 3A). In contrast, cortices kept under similar but static conditions were characterized by massive cell death (Fig. 4B) as compared to cortices subjected to shaking (Fig. 4A).

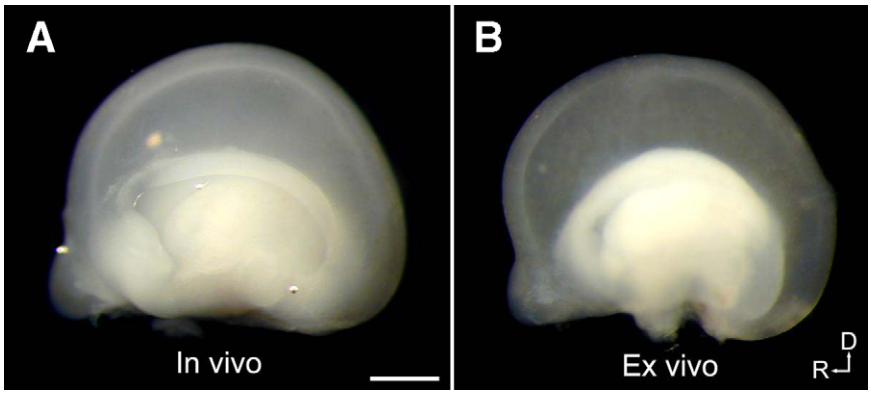

Fig. 3. Cortices cultured *ex vivo* maintain a healthy morphology. Whole mount views of a freshly isolated E15 cortex (A) and an E14 cortex after 24 h of culture (B). Note that there is no significant change in gross cortical morphology following *ex vivo* culture. D: dorsal; R: rostral. Scale bar, 0.5 mm.

3.2. Cortical compartments and mitosis are preserved in ex vivo culture

The cortical region immediately adjacent to the lateral ventricle, termed the ventricular zone (VZ) contains proliferating neural progenitors cells (NPCs) in various stages of the cell cycle. Immunolabeling for Ki67, a marker for proliferating cells, revealed VZ limits that were similar between *in vivo* and *ex vivo* preparations (Fig. 5A and B). These NPCs undergo interkinetic nuclear migration whereby the nucleus migrates between the top and bottom of the ventricular zone (VZ), dependent upon its cell cycle phase (Sauer, 1935). Analysis of migrating cells using bromodeoxyuridine (BrdU) pulses showed that this interkinetic migration was preserved in *ex vivo* cultures (Kingsbury et al., 2003). Examination of mitotic cells using an antibody against the phosphorylated form of histone H3 (Hendzel et al., 1997) showed that mitotic cells in the *ex vivo* culture were dividing at the bottom of the VZ (Fig. 5D), similar to cells *in vivo* (Fig. 5C). Furthermore, there were no significant differences in the percentage of mitotic cells ($P = 0.74$, unpaired *t*-test) or cerebral wall thickness ($P = 0.08$, unpaired *t*-test) between E15 freshly isolated cortices (i.e. *in vivo* cortices) and E14 cortices cultured for 24 h *ex vivo*.

3.3. Migration and differentiation are preserved in ex vivo culture

The correct migration and position of cortical cells within the cortex is essential for proper brain function. When neural progenitor cells exit the cell cycle, they can be identified by the marker Tuj-1, otherwise known as neuronal class III β-tubulin (Menezes and Luskin, 1994). Upon migrating out of the VZ, class III β-tubulin positive neurons settle in the developing cortical plate (CP). To examine whether neuronal migration was preserved in the *ex vivo* culture, class III β-tubulin labeling in the CP was examined. Within this cortical region, the num-

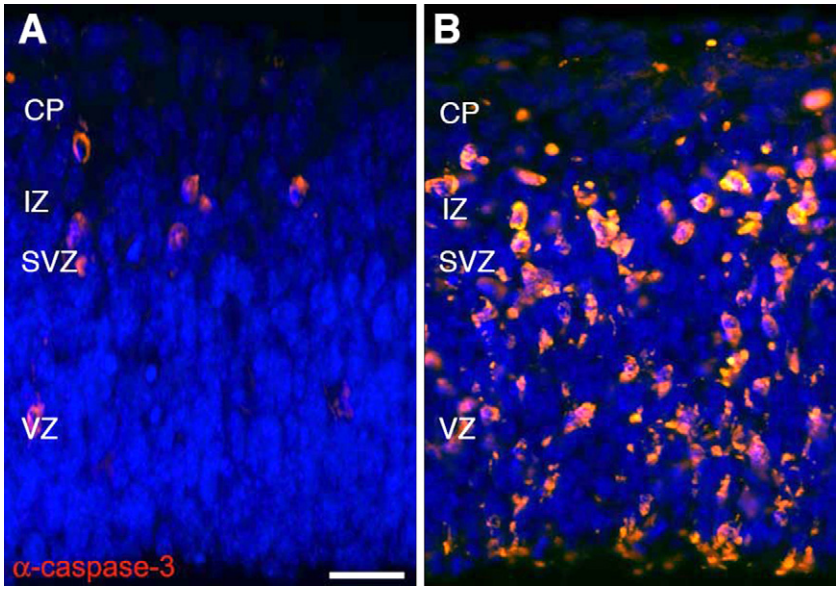

Fig. 4. Orbital shaking of *ex vivo* cultures prevents cell death. Matched E14 cortices were shaken at 70 rpm (A) or not shaken (B) for 24 h *ex vivo* and immunostained for cleaved caspase-3 (red). Individual nuclei are stained blue with DAPI. Note that the cortex from the static culture shows numerous caspase-3 positive cells, while the shaken cortex shows very few. CP: cortical plate; IZ: intermediate zone; SVZ: subventricular zone; VZ: ventricular zone. Scale bar, 25 μm.

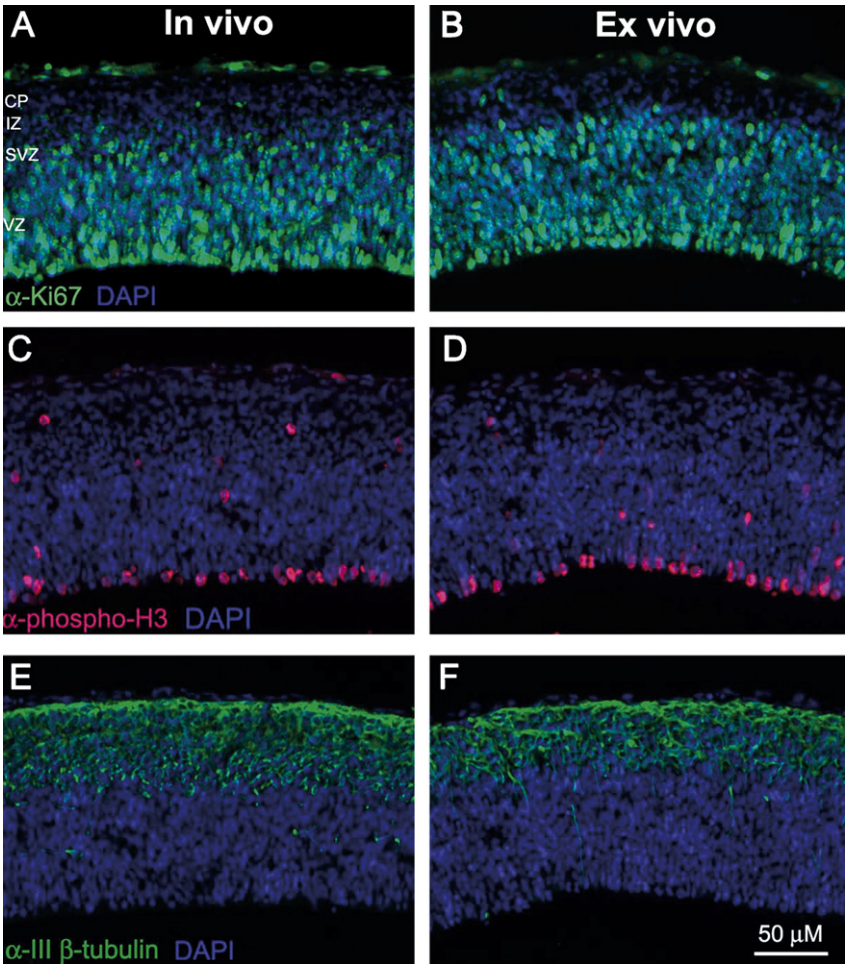

Fig. 5. *In vivo* cortical compartments, mitosis and differentiation are preserved in cultured cortices. Cortices were either freshly excised at E15 (A, C and E) or isolated at E14 and cultured for 24 h (B, D and F). VZ limits are similar in both preparations, as identified by immunolabeling for Ki-67, a marker of proliferating cells (A and B). The location of mitotic cells, identified by immunolabeling for phospho-histone H3, is comparable between *in vivo* (C) and *ex vivo* conditions (D). The number and position of early postmitotic neurons in the cortical plate of both *in vivo* (E) and *ex vivo* (F) preparations identified by neuronal class III β-tubulin labeling, indicate that differentiation and migration are preserved in the *ex vivo* system. Scale bar, 50 μm.

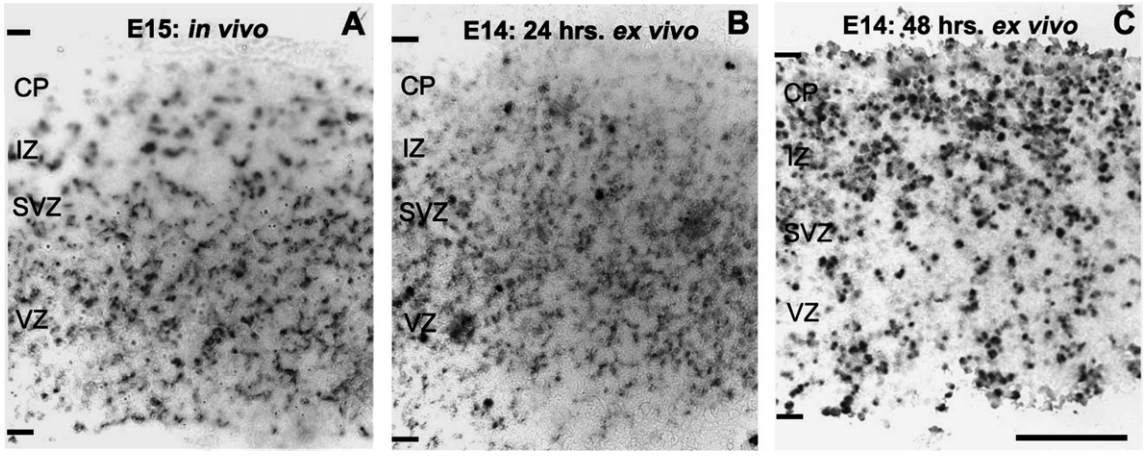

Fig. 6. A comparison of cell death in E15 freshly isolated cortices and E14 cortices cultured for 24 and 48 h. No significant difference was observed in the percentage of ISEL$^+$-labeled cells at E15 *in vivo* (A) vs. E14 cortices cultured for 24 h (B). In contrast, the number of dying cells labeled by ISEL$^+$ was markedly increased in E14 cortices cultured for 48 h (C). Scale bar, 50 μm.

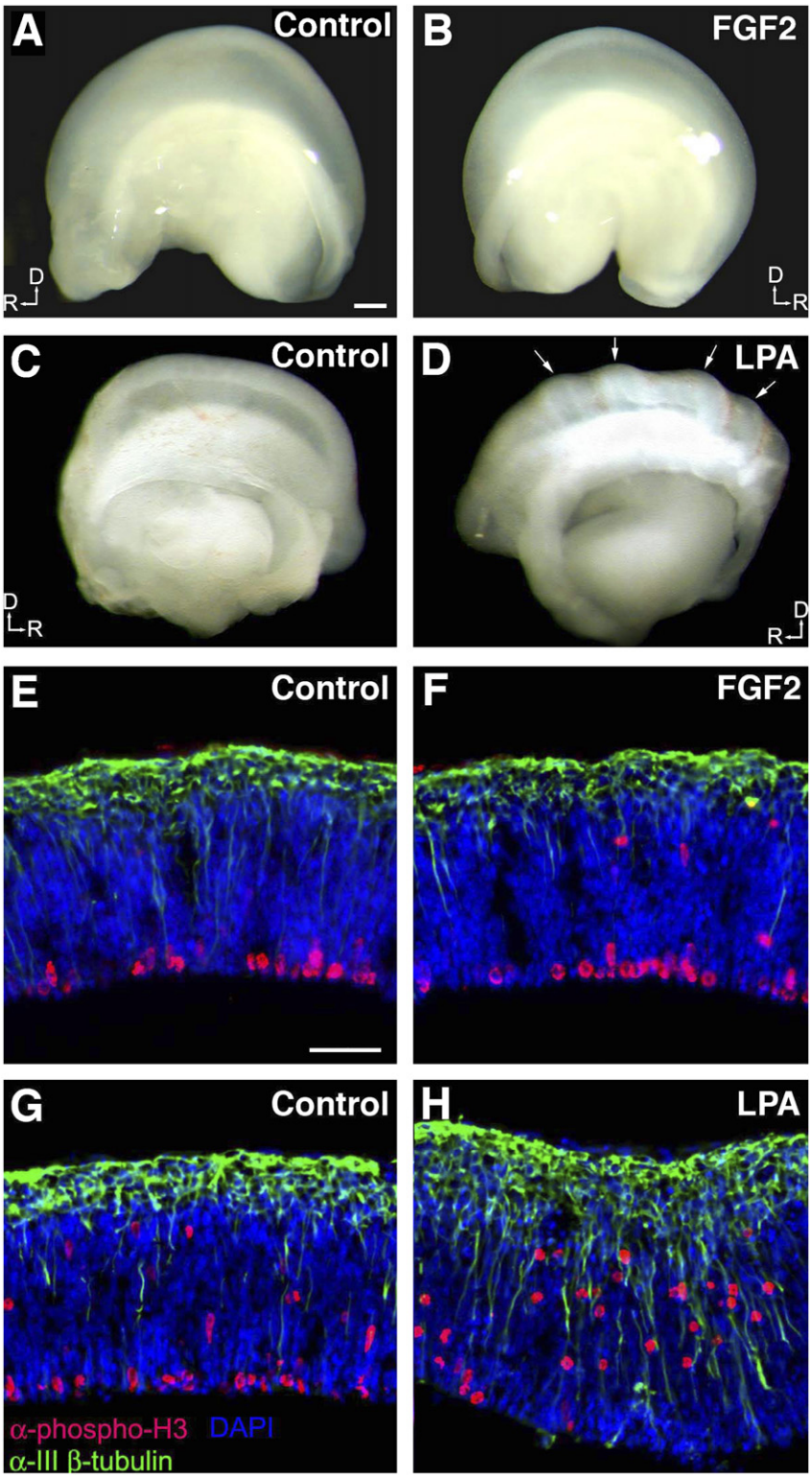

Fig. 7. The *ex vivo* model can be used to screen growth factors for specific effects on cortical morphology and neurogenic processes. Compared to control cortices, exogenous exposure to FGF2 has no effect on cortical morphology (A and B) whereas LPA causes pronounced cortical folds that resemble gyri (arrows) and sulci (C and D). Scale bar, 0.25 mm. Correspondingly, FGF2 produces no change in the appearance of mitotic figures (red) or differentiated neurons (green) relative to untreated controls (E and F). In contrast, LPA causes an increase in the number of mitotic figures, displacement of mitotic figures from the bottom of the VZ and an increase in terminally differentiated neurons (G and H). Scale bar, 50 μm.

ber of class III β-tubulin labeled cells was similar for both *ex vivo* and *in vivo* conditions (Fig. 5E and F; $P = 0.56$, unpaired *t*-test).

3.4. Cell death in ex vivo cortices is similar to that in vivo

The developing cerebral wall is characterized by a substantial amount of proliferative cell death (Blaschke et al., 1996, 1998), that when altered, can have profound effects on brain formation (Kuida et al., 1998, 1996; Kuster et al., 1993; Li et al., 2003; Pompeiano et al., 2000). To examine whether *in vivo* cell death was preserved in *ex vivo* cortices, *in situ* end-labeling plus ($ISEL^+$) labeling was used to label dying cells throughout the cerebral wall (Blaschke et al., 1996). The number of $ISEL^+$-positive cells was similar between E15 freshly isolated cortices and E14 cortices cultured for 24 h (Fig. 6A and B; E15 freshly isolated cortices, 64% ± 7.0; E14 cultured cortices, 66% ± 7.5; $P = 0.89$, unpaired *t*-test), indicating that 24 h of explant culture does not alter normal *in vivo* cell death. Note that the 64% cell death observed *in vivo* at E15 is in agreement with previously published accounts of cell death at E14 (∼55%) and E16 (∼75%) (see Fig. 4B and 9 from Blaschke et al., 1996). Extending culture time beyond 24 h using the current parameters (i.e. Opti-MEM I medium plus supplements, 5% CO_2 level, etc.) resulted in a substantial increase in cell death at 36 h (data not shown) and 48 h (Fig. 6C). At the later time point, the entire cerebral wall displayed massive cell death and reduced thickness (compare Fig. 6C with B), demonstrating that the explants were compromised after 2 days in culture.

3.5. Extrinsic factors induce cortical folding in ex vivo culture

To test the feasibility of using free-floating *ex vivo* brains to identify potential factors affecting brain anatomy, the two cerebral hemispheres from each animal were physically separated so that one could be cultured with an extrinsic factor while the other was cultured in control medium (Fig. 2). E14 cortices were used since this age is the midpoint of mouse cortical neurogenesis (Caviness, 1982). One hundred percent of the hemispheres exposed to LPA ($N = 13$) but not FGF2 displayed significant and obvious cortical folding compared to opposite hemispheres obtained from the same animals that were cultured in control medium (Fig. 7A–D; Kingsbury et al., 2003). In addition, after 24 h, neurogenic processes such as mitosis and differentiation were altered following LPA but not FGF2 treatment (Fig. 7E–H; Kingsbury et al., 2003).

4. Discussion

Since the beginning of the last century, scientists have been looking for new ways to culture cells and tissues (Strangeways and Fell, 1926). As with all *in vitro* systems, both advantages and potential limitations exist. In the *ex vivo* cortical culture method described here, the processes of mitosis, differentiation and migration of young postmitotic neurons were similar to those described *in vivo* within the defined period of culturing. Furthermore, this paradigm revealed dramatic anatomical and histological alterations of the embryonic cerebral cortex after specific growth factor treatments, thus serving as a gain-of-function model. A possible limitation of the system is poor viability after extended culture time (>24 h). Whereas cell death was similar between E15 *in vivo* tissue and E14 cortices cultured for 24 h, substantial cell death was seen after 36 and 48 h of culture; however, this may be improved with different growth media. Still, for many applications, the advantages of this *ex vivo* system outweigh the potential limitations.

Our free-floating cerebral cortex approach provides a controlled and precise way of varying ligand type, concentration, exposure time and other variables for samples derived from any genotype, under identical conditions, using tissue from the same animal (i.e. comparing one hemisphere to the other) (Kingsbury et al., 2003, 2004). In conjunction with animal studies (e.g. knockout mice), this system can provide important basic information about normal brain formation and also reveal new roles for biological molecules, which may regulate brain morphology and processes such as cortical folding.

A critical aspect of this approach relies on orbital shaking while culturing whole cerebral cortex hemispheres. Moscona and colleagues started using orbital shaking to culture aggregates of cells more than 40 years ago (Moscona, 1961), and more recently we, among others, have used this technique to culture retinal explants (Linden et al., 1999; Rehen et al., 1999, 1996). We believe that orbital shaking provides better aeration and diffusion of nutrients *in vitro*, which vastly improves the viability of intact cerebral cortices in culture.

We recently used this *ex vivo* cortical culture method to study the effects of lipid molecules on cortical architecture by examining the effects of exogenously applied LPA. Within 17 h, LPA induced cortical folding and reduced cell death while increasing cell cycle exit of NPCs in the embryonic cortex (Kingsbury et al., 2003). Moreover, this process was shown to involve two LPA receptors, LPA_1 and LPA_2, based on the use of cortices from LPA_1LPA_2 receptor double null mice. By contrast, cortical folding was not observed using the well-studied growth factor FGF2. These results are particularly intriguing given that cortical folding malformations are associated with mental retardation and epilepsy (Crino, 2004; Ertl-Wagner et al., 2003; Guerrini et al., 2003). Examples of other diseases associated with alteration in cortical folds include lissencephaly, in which the normally convoluted cerebral cortex is smooth (Olson and Walsh, 2002; Pilz et al., 2002; Reiner, 1999), autism, and schizophrenia, in which an increase in the amount of cortical convolutions is observed (Hardan et al., 2004; Harris et al., 2004; Narr et al., 2004). It is very likely that other portions of the embryonic CNS can also be similarly cultured. This *ex vivo* culture method thus offers a new approach to studying and manipulating the cellular and molecular mechanisms influencing brain physiology and pathology.

Competing interests statement

The authors declare that they have no competing financial interests.

Acknowledgements

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