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Modeling the Impact of Alcohol on Cortical Development in a Dish: Strategies From Mapping Neural Stem Cell Fate

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Summary

During the second trimester period, neuroepithelial stem cells give birth to millions of new neuroblasts, which migrate away from their germinal zones to populate the developing brain and terminally differentiate into neurons. During this period, large numbers of cells are also eliminated by programmed cell death. Therefore, the second trimester constitutes an important critical period for neuronal proliferation, migration, differentiation and apoptosis. Substantial evidence indicates that teratogens like ethanol can interfere with neuronal maturation. However, there is a paucity of good model systems to study early, second trimester events. In vivo models are inherently interpretatively complex because cell proliferation, migration, differentiation, and death mechanisms occur concurrently in regions like the cerebral cortex. This temporal overlap of multiple developmental critical periods makes it difficult to evaluate the relative vulnerability of any individual critical period. Our laboratory has elected to utilize fetal rodent cerebral cortical-derived neurosphere cultures as an experimental model of the second-trimester ventricular neuroepithelium. This model has enabled us to use flow cytometric approaches to identify neuroepithelial stem cell and progenitor sub-populations and to show that ethanol accelerates the maturation of neural stem cells. We have also developed a simplified mitogen-withdrawal/matrix-adhesion paradigm to model the exit of neuroepithelial cells from the ventricular zone towards the subventricular zone and cortical plate, and their maturation into multipolar neurons. We can treat neurosphere cultures with ethanol to mimic exposure during the period of neuroepithelial proliferation and by using the step-wise maturation model, ask questions about the impact of prior ethanol exposure on the subsequent maturation of neurons as they migrate and undergo terminal differentiation. The combination of neurosphere culture and stepwise maturation models will enable us to dissect out the contributions of specific developmental critical periods to the overall teratology of a drug of abuse like ethanol.

Keywords

Neural stem cells; CD133; Sca-1; ABCG2; c-kit; neurosphere culture; Mitogen; FGF; EGF; LIF

1 Introduction

1.1 The Fetal Alcohol Syndrome

Heavy ethanol consumption during pregnancy can persistently alter fetal development and lead to a constellation of craniofacial, brain, and cardiovascular defects that are collectively termed the fetal alcohol syndrome or, F.A.S (1). The constellation of brain defects includes microencephaly, malformations of gyri, diminution or loss of interhemispheric communicating fiber tracts like the corpus callosum (reviewed in (2,3)), and the presence of “brain warts” or heterotopias containing displaced neurons (3,4). Because of an increasing recognition that lower levels of ethanol consumption during pregnancy also can lead to

neurological, behavioral, and cognitive deficits, the range of defects associated with *in utero* alcohol exposure have collectively been termed fetal alcohol spectrum disorders or, F.A.S.D (5,6).

In utero alcohol exposure is the most important nongenetic cause of mental retardation. Genetic susceptibility factors do increase vulnerability to *in utero* ethanol exposure. However, the identified risk factors include genes like alcohol dehydrogenase (e.g., the, A. D.H1B*2 allele (7)), which control the metabolism of ethanol and, consequently, titrate either maternal or fetal blood alcohol levels. It follows, therefore, that a dose of ethanol that results in permanent alteration of the nervous system must produce its effects by disrupting events underlying critical periods of neural development, rather than recruiting genetic susceptibility factors per se. In other words, the effect of ethanol during a particular developmental period is determined by the specific biological events that occur during that period. Therefore, to study the effects of ethanol on brain development, we need to pay close attention to appropriately modeling relevant underlying biological events. It is critically important that models of neural development closely mimic pertinent aspects of *in utero* developmental biology.

1.2 Effects of Alcohol During Brain Growth and Development

The brain growth spurt period (the second and third trimester equivalent of human gestation, comparable with the latter half of gestation and the early postnatal period of rodent development (8)), is characterized by rapid neuronogenesis ((9), the initial period of neuron generation), compensatory apoptosis (10,11), neuroblast migration out of the ventricular zone (VZ), and early neuronal maturation. This developmental phase constitutes a period of particular vulnerability to alcohol. Most studies on the effects of alcohol on brain development have focused on the third-trimester model. For example, early studies from West and his colleagues used a third-trimester model in rats to demonstrate neuronal cell loss in the cerebellum, olfactory bulb, and hippocampus after alcohol exposure (12–17). Rodent cell culture models and tumor-derived cell lines also have been used extensively to understand the effects of ethanol on neuronal survival and differentiation, i.e., events that occur during the third trimester. Using these models, we and others, have shown that, in differentiated neural tissue, part of alcohol's neurotoxicity may be caused by the induction of death mechanisms (18–21), the loss of growth and survival factors (22–28), alterations in neuronal migration (29), and in neurotransmitters systems (30), among others. Further, in vitro studies found that the state of differentiation of the cells determined the degree of sensitivity to alcohol insult (31,32), confirming in vivo work showing windows of vulnerability that reflected maturation of the neurons (16,33,34).

In contrast to the extensive research that has focused on the third-trimester effects of ethanol, the second trimester represents a poorly understood and lessstudied period of vulnerability to ethanol. The second trimester is an important time frame for study of alcohol's effects because during this period, millions of new neurons are born, migrate away from neuroepithelial germinal zones (primarily the ventricular and subventricular zones), and populate various brain regions (35,36), laying down a cellular framework for the rest of brain development. The maturation of neural stem cells plays a crucial role in the process of neuronogenesis. An early study by Barnes and Walker (37) reported a loss of hippocampal neurons from a second-trimester equivalent alcohol exposure, and significant work from Miller's lab showed alcohol-induced changes in cortical neuronogenesis (38–40). Others have shown that rats exposure to ethanol over a 2-d window from, G. D.14–15 exhibit an immediate enlargement of the, S.V.Z, suggesting, N.S.C/NPC maturation (41), disorganized cortical architecture at the end of the neuronogenic period (42), and a persistent thinning of lamina V of the rodent cerebral cortex (43), suggesting that the effects of second-trimester ethanol exposure are persistent.

Until recently, we have lacked good culture models to examine the cell and molecular biological underpinnings of ethanol's actions. In this chapter, we will discuss cell and tissue culture models that we use in our laboratory to model pertinent events that occur during the second trimester of human gestation, with specific reference to the formation of the cerebral cortex.

1.3 Neurodevelopment During the Second-Trimester Period

During the second trimester-equivalent prenatal period of neurogenesis, the number of neuroepithelial cells expands rapidly to generate most of the neurons of the adult brain (44) requiring, as with other tissues (Fig. 1. (45)), the conversion of uncommitted stem cells to more fate-restricted progenitors, then to blast-cells, and ultimately neurons (Fig. 2). The fetal murine cerebral cortical ventricular zone initiates proliferation by gestational day (GD) 11, and a neural stem cell (NSC) that starts proliferating on, G. D.11 will undergo ~11 integer cell cycles during the period required to generate cortical plate (CP) neurons (36). During the peak period of neuronogenesis, the rodent cerebral cortex is estimated to add ~2400 new neurons to the cerebral CP per minute (46). Neuroepithelial cells are coupled to each other by gap junctions during S and G2 phases of cell cycle (47) and are vulnerable to apoptosis during these cell cycle phases (11). Consequently, the fate of a single neuroepithelial cell is likely to be tied to the fate of its neighbors, and to its cell cycle stage, and the collective neuroepithelial response to a teratogen may be more critical than the response of individual neuroepithelial cells. A teratogen that alters the rate of proliferation and death in neuroepithelial cells is likely to significantly alter the structure of the mature brain.

1.4 The Neurosphere Culture Model Recapitulates the Second-Trimester Neuroepithelium

The nonuniform distribution of cell proliferation cycles throughout the neuronogenic period suggests that classes of rapidly proliferating neuroepithelial progenitors that are present early in the neuronogenic period are either eliminated or otherwise suppressed as the ventricular zone matures. We and others have modeled the fetal neuroepithelium using neurosphere cultures derived from the fetal mouse and rat ventricular zones. We typically isolate cortical tissue from GD 12.5 mouse fetuses corresponding to the initial period of CP neuronogenesis (although we have also isolated tissue from rat fetuses and, at later gestational ages, corresponding to the peak of CP neurogenesis). Isolated cells are dispersed into defined culture medium and may be further fractionated by immunomagnetic separation (MACS, Miltenyi Biotech), or by fluorescence-assisted cell sorting (FACS). In the presence of mitogenic factors, the individual neuroepithelial cells form floating clonal colonies, or spheroid bodies. In the absence of extracellular matrix molecules, cells preferentially adhere to each other and are often referred to as "neurospheres." An individual neurosphere is quite heterogenous, with respect to maturation state. Cells expressing the nestin, glial fibrillary acidic protein (GFAP), and the early neuronal maturation marker, microtubule-associated protein-2 (MAP-2), can all be identified within neuronal cultures. However, other markers such as NeuN, which identify mature neurons, are not present in neurosphere cultures. Neurospheres grown from single cells can assume varying sizes during a 48- to 72-h period, suggesting that their parental neuroepithelial cells are intrinsically variable with respect to cell cycle kinetics, perhaps reflecting an emerging heterogeneity of fate within the neuroepithelium.

1.5 Mapping the Diversity of Fetal Neuroepithelial Cells

Increasing evidence suggests that cortical neuron heterogeneity results from early diversification of the neuroepithelium (48–52), well before the advent of external influences in the form of cortico-fugal projections from other brain nuclei. For example, pyramidal neurons of the mature cerebral cortex are generated within the cortical ventricular zone,

wherea interneurons are generated within the ventral ganglionic eminences and migrate tangentially into the CP (53–55). Neuronal heterogeneity in terms of gene expression patterns and lamina preference emerges within each neuroepithelial zone as well. Transcription factors like Lhx-1&2, TBr-1, and Emx-1 identify distinct subpopulations of neuronal precursors (53,56). Because of these and other data, it has been suggested that the cortical neuroepithelium contains a “protomap” (56) of the mature adult cortex and that the principal determinants of cortical structure arise from diversification of stem and progenitor cells within the cortical neuroepithelium itself.

Transcription factor identity, however, is not useful for isolating live neuroepithelial subpopulations. Such cell-types are more easily isolated based on their differential expression of cell-surface antigens. For example, we and others have shown that cell-surface molecules like the Fas/Apo-1/CD95 suicide receptor (10,11), β2-microglobulin, a, M. H.C class I antigen (50), and receptor tyrosine kinase, EphA (56), all mark subsets of neuroepithelial precursors within the, V. Z. Interestingly, these proteins (57,58), or their family members (59), also mark cells in various stages of hematopoiesis, supporting the notion that stem and progenitor cells in dissimilar tissues nevertheless express a common repertoire of surface antigens.

Hematopoietic-derived cells express different surface antigens at various developmental transitions (60) and, thus, surface antigen expression reflects the status of development along a competency continuum. In our research, we have elected to use cell-surface markers derived from the hematopoietic system, like, C. D.133/prominin-1, Sca-1 (Ly6A/E), CD117/c-kit, and, A. B.CG2 (ATP-binding cassette, sub-family G (WHITE), member 2), to define early cortical neuroepithelial subpopulations. These cell surface markers have been used successfully to monitor stem cell heterogeneity in a variety of tissues (61–64). ABCG2 in particular is most likely to mark stem cells uniquely. Stem cells in the hematopoietic and nervous system (61,65,66) display a unique ability to rapidly induce efflux of Hoechst dye (#33342), thereby generating a characteristic staining-pattern (termed the side population or ‘SP’), which can be assessed by flow cytometry (67,68). The, S. P.-population in hematopoietic tissues represents less than 1% of the total population and is a rare event, as is expected for a stem cell group. The protein, ABCG2 (69), confers a, S. P.-population phenotype to stem cells, and is downregulated in more differentiated cells (70–72). In our research, we have therefore used, A. B.CG2 expression to mark neural stem cells, and to monitor this population after ethanol exposure. Stem cells can exhibit two alternate modes of cell division (73); the first, symmetric division (Fig. 1a), results in the formation of two daughter stem cells, whereas the second, i.e., asymmetric division, results in the formation of one daughter stem cell, and a second, more mature, daughter progenitor cell (Fig. 1b). Symmetric division boosts the pool of stem cells while asymmetric division maintains the stem cell pool.

In the mouse, a major burst of cell proliferation occurs between, G.D.11 and, G.D.14, encompassing 63% of the integer cell cycles that span the neuronogenic period (36). Within the hematopoietic system, this burst pattern is consistent with the appearance of colony-forming units (CFUs or early progenitors), which proliferate rapidly by symmetric division (Fig. 1b) to regenerate lineage-specific colonies, and are present in large numbers. Recent evidence indicates that such symmetric division of neural, C. F.U-equivalents occurs at a high rate in the apical portion of the fetal neuroepithelium, and is an important source of neurons (73). Our data show that the antibodies to Sca-1, CD133, and, C.D.117 label ~30%, 12%, and 20% of neuroepithelial cells, respectively, by flow cytometry (74). Because these antigens mark a significantly larger number of cells than, A. B.CG1, it is likely that these antigens mark more mature (CFU and later) stages of neuronal lineage. Later symmetric

division may result in the continued formation of lineage committed, blast precursors (Fig. 1c).

1.6 Modeling Early Cortical Neuronal Differentiation With Culture Models

Cells leave the, V.Z. to directly form CP neurons (75), or to form radial glial and astrocytic intermediate blast precursors to additional neurons (Fig. 2 (76,77)). Our laboratory has been interested in developing models to study this transition between cell proliferation and differentiation in the cerebral cortex. In our initial model of cortical maturation, we infected fetal rodent-derived cortical cells with an adenovirus expressing a temperature sensitive, S.V.40-large T antigen (*ts*TA) (78). At the permissive temperature for T-antigen expression (+*ts*TA) fetal cortical cells proliferate rapidly, assume an epithelioid morphology, and express the intermediate filament protein, nestin. However, at the non-permissive temperature for T-antigen expression (−*ts*TA), neuroepithelial cells exit cell cycle, and large numbers of cells undergo apoptosis by p53-dependent mechanisms (11,78). Surviving cells, however, undergo morphological transformation and initiate the growth of neurites. Treatment with retinoic acid results in further differentiation along a neuronal lineage (78). In this model, SV40-*ts*TA served as a molecular switch between states of cell proliferation on one hand, and differentiation and apoptosis on the other. Although this model did partially segregate critical periods, it was subject to several limitations. First, it was not clear whether specific subpopulations of the neuroepithelium are selectively eliminated by apoptosis in the −*ts*TA condition. Second, the, S.V.40-T antigen maintains cells in cell cycle by suppressing cell cycle arrest factors like p53 and p21/Waf-1, thereby permitting the accumulation of gene mutations and ultimately, escape from cell cycle controls.

1.6.1 Naturalistic Models of Neuronal Differentiation—In comparison with, S.V.40-T antigen-transformed cells, neurosphere cultures can be differentiated under well-defined, naturalistic conditions, to model the exit of neuroblasts out of the, V.Z., and their maturation into multipolar neurons. In the model outlined herein, we empirically defined conditions that include sequential withdrawal of mitogenic factors and addition of extra-cellular matrix (to activate integrin signaling (79)), resulting in the sequential appearance of two unique morphological phenotypes of migratory bipolar, or multipolar neurons. We classify the migratory, bipolar cells as belonging to the subventricular zone (SVZ) or “early-neuronal differentiation” phenotype. The multipolar morphology is characteristic of both, S.V.Z (75) and CP neurons. We refer to this phenotype as the, C. P. or “late neuronal differentiation” phenotype. This model provides for several advantages. We have shown that the transformation between proliferation and differentiation phenotypes is not accompanied by a significant alteration in apoptosis (80), unlike our previous, S. V.40-T antigen-transformed model. Second, this model recapitulates the major second-trimester events, neuroepithelial proliferation, migration, and neuronal maturation, which are necessary for the development of brain regions like the cerebral cortex. Finally, and most importantly, this model permits us to expose cells to a presumptive teratogen during one stage of differentiation, and to examine the immediate (activational) effects on cells within that stage, as well as the persistent (organizational) effects of that teratogen on subsequent neuronal differentiation. This model therefore enables us to capture the key feature of a teratogen, which is its ability to persistently and permanently alter the development of tissues, even though it is no longer present within that tissue’s environmental milieu.

2 Materials

Dulbecco’s phosphate-buffered saline (DPBS, cat. no. 14040-133), Dulbecco’s Minimal Essential Medium (DMEM, cat. no. 10313-021) Hanks’s Balanced Salt Solution (HBSS, cat. no. 14175-095), trypsin/EDTA (cat. no. 25300-112), bovine serum albumin, fraction-V,

(BSA-V, cat. no. 1526037), Laminin (cat. no. 23017-015), ethylene diamine tetraacetic acid (EDTA), DMEM/F12 (cat. no. 11330-032), basic fibroblast growth factor (bFGF; cat. no. 13256-029), human recombinant epidermal growth factor (EGF; cat. no. 53003-018) ITS-X supplement (Insulin, Transferrin, Selenium, cat. no. 51500-056), and heparin (cat. no. 15077-019) are obtained from Invitrogen. Recombinant human Leukemia Inhibitory Factor (LIF; cat. no. L200) is obtained from Alomone Labs. Progesterone (cat. no. P6149) and 95% ethanol is obtained from Sigma.

3 Methods

3.1 Isolation of Embryonic Neural Precursors

Timed-pregnant C57/Bl6 mice were generated by timed-mating for 1 h, and GD −0.5 was defined as the day when female mice exhibited postcoital sperm plugs. Mice were maintained in the animal housing facility at Texas A&M University System Health Sciences Center, College of Medicine, on a **12,** 12-h light-dark schedule. At, G. D. 12.5, mice were anesthetized with a mixture of ketamine (0.09 mg/g body weight) and xylazine (0.106 mg/g body weight) by intramuscular injection. The abdomen of the anesthetized pregnant mouse was swabbed first with 80% ethanol (vol/vol), and then with Betadine (10% povidone-iodine; *see* Note 1). A laparotomy was then performed with fresh sets of dissection instruments to make sequential incisions into the skin and underlying peritoneum. The gravid uterus was dissected, rinsed in chilled, D. P.BS (*see* Note 2), the fetuses dissected, and fetal brains removed and placed in chilled, H. B.SS supplemented with glucose and magnesium chloride. Meningeal tissue was removed (*see* Note 3), regions of the mouse fetal brain corresponding to the structural precursor of the neocortex were isolated, and care was taken to exclude the structural precursors to the striatum and hippocampus. Individual cortical fragments (*see* Note 4) are collected in sterile 15-mL conical tubes and gently triturated in trypsin/EDTA. Trypsin is inactivated with, D. M.EM containing 10% fetal bovine serum. The cell suspension is centrifuged for 5 min at 18°C, 1000 rpm (300 *g*). Cell pellets are resuspended in chilled, D. P. BS containing 0.5% BSA, Fraction-V, and 2.0 m*M*

[1]Betadine (10% povidone-iodine) can be purchased as an over-the-counter antimicrobial agent from any local pharmacy or supermarket. It is easy to apply if you first drench the mouse abdomen with 80% ethanol, to wet the fur. Apply Betadine liberally, ensuring coverage of the entire lower abdomen, including the ano-genital region and the proximal tail. Wait 5 min to let the Betadine dry, then use a separate pairs of scissors and forceps to open up the skin and underlying peritoneum so to limit the carryover of contamination from one layer to the next.

[2]While dissecting out the uterus, be careful to prevent contact with the retracted skin and peritoneal flap. Avoid perforating the gastrointestinal system while dissecting away the uterine horns, to prevent bacterial contamination. This dissection may be performed under aseptic conditions. Place the dissected uterus into a Petri dish containing sterile DPBS and transfer the Petri dish to a sterile laminar-flow hood or workbench. Rinse out the uterine horns by three transfers to new PBS-filled Petri dishes. Serial rinsing serves to eliminate any bacteria or fungal spores that become adherent to the uterine horns during the initial dissection. The uterine horns can then be sliced open with a fresh pair of scissors, and the fetal amniotic sacs should then be extracted and transferred to a new PBS-filled Petri dish. Using a fresh set of Dumont-style forceps, dissect the individual fetuses away from their amniotic sacs and placenta, and transfer them to a new HBSS-filled Petri dish. Further microdissections are performed on a chilled stage of a dissecting microscope, within the confines of a sterile, laminar-flow workbench. The stage is chilled to 4°C by circulating a chilled mixture of polyethylene glycol and water through channels imbedded into the stage. Using a fresh set of microscissors and forceps, the fetal cranial skin is removed. At this developmental stage, the calvarial bones are extremely thin and can be dissected away with scissors and microforceps. Cuts are made at the level of the olfactory bulb and the brainstem, and the brain lifted away. The key to successful sterile dissection is to serially dilute bacterial and fungal inoculums by repeated changes of sterile PBS. The use of fresh, sterile sets of instruments at each step of the dissection also limits carry-over of contaminants.

[3]It is easier to start peeling away meningeal tissue from the ventral portion of the brain first. Using a pair of fine, Dumont-style forceps, flip the brain over so that the ventral surface faces upwards. Gently peel back the meningeal tissue from around the hypothalamic region first, then working outwards, peel meningeal tissue as a continuous sheet over the lateral margins of the telencaphalic vesicles. Then flip the brain over so that the dorsal surface faces upwards. Continue peeling back the meningeal tissue gently over the telencephalic vesicles so as not to disrupt the meningeal sheet. The cortical neuroepithelium is fragile at this age, and is easily fragmented by careless handling. We find that trans-illumination with a flat panel optic fiber light, set into the cooling plate below the brain tissue, serves to increase the contrast between meningeal and brain tissue. Structures of interest, tissue precursors to the cortex, hippocampus, striatum and diencephalon, for example, can then be microdissected with a microscalpel.

[4]To limit the impact of litter-to-litter variations on studies, we typically collate fetal cortical tissue from three to four litters per experiment into a single collection tube

EDTA. Total cell counts are determined using a hemocytometer. Dispersed neuroepithelial precursors are established in culture at an initial density of 10^6 cells in T-25 flasks containing serum-free mitogenic media (DMEM/F12, 20 ng/mL bFGF, 20 ng/mL EGF, 0.15 ng/mL (*see* Note 5) LIF, ITS-X supplement, 0.85 Units/mL heparin, and 20 n*M* progesterone). Cultures are incubated at 37°C, 5% CO_2 in a humidified environment to generate neurospheres (Fig. 3). Cells are allowed to proliferate as neurospheres until cultures achieved a density of 2×10^6 cells per T25 flask (*see* Note 6), and after approximately 6–8 passages (at ~3 d per passage), used for experiments.

3.2 Ethanol Treatment

Neurosphere cultures in T25 flasks are randomly assigned to control or ethanol treatment groups. In our laboratory, we have treated neurosphere cultures with a wide range of ethanol doses, ranging from 60 mg/dL (~13 m*M*) to 920 mg/dL (200 m*M*). We monitor culture media ethanol concentrations with gas chromatography. Ethanol containing medium is prepared freshly before use, from 95% ethanol. Each flask is defined as a single sample. Culture medium is changed every 2 d. Control and ethanol-treated flasks are capped tightly with phenolic caps, and sealed with parafilm to limit the loss of ethanol. These measured doses range in equivalence to consumption levels that can be attained by social drinkers (60 mg/dL) to those attained by chronic alcoholics (320–620 mg/dL, 70–135 m*M* (81,82)), to levels above those typically attainable in chronic alcoholics (920 mg/dL). Our analysis indicates that there is no significant change in ethanol content within culture dishes over the exposure time period (Fig. 4).

3.3 Differentiation of Neurosphere Cultures

We characterize our neurosphere cultures as a fetal ventricular-zone “ neuroepithelial proliferation” or, V. Z. model. To initiate the differentiation program, neurosphere cultures are transferred to fresh T-25 flasks, Petri-dishes, or microwell plates coated with laminin (at 50 ∝g/mL in, D.M.EM/F12 for 1 h). The presence of laminin by itself is enough to cause neurospheres to become adherent and permits neuroepithelial cells to migrate away from the parent neurosphere (80), indicating that laminin-mediated activation of integrins is a strong migratory stimulus. However, migrating neuroepithelial cells acquire a squamous epithelioid appearance and do not exhibit neurites and growth cones that are typical of migratory neurons. Within 24 h after concurrent removal of EGF and LIF from the culture medium (i.e., the +Laminin/+FGF/−EGF/−LIF condition) these migratory cells lose their epithelioid morphology and assume a bipolar appearance (Fig. 5). Unlike cells of the neurosphere cultures, these migrating cells express the neuronal nuclear antigen, NeuN, within their nuclei and consequently can be defined as early, migratory neurons. We refer to the +Laminin/+FGF/−EGF/−LIF condition as the ‘Early Neuronal Differentiation’ or SVZ model condition.

Neuroepithelial cells cultured on Laminin, without any mitogenic factors (i.e., in the +Laminin/−FGF/−EGF/−LIF condition), continue to express NeuN in their nuclei, and additionally, assume a multipolar morphology, characteristic of more mature neurons. We refer to this condition as the “late neuronal differentiation condition” or the CP model. Neurosphere cultures can be directly transferred to either the SVZ or CP conditions to produce the early or late neuronal phenotypes or, alternatively, differentiated sequentially through the, S. V.Z and, C. P. condition. We typically culture cells in these early or late

[5]The concentration of LIF at 0.15 ng/mL (as with other factors) has been empirically determined in our laboratory. In our hands, a 10-fold increase in LIF concentration (i.e., 1.5 ng/mL) results in neurosphere cultures becoming adherent to the culture plates, suggesting that greater levels of LIF induce morphological transformation.

[6]Cell number can be verified using a standard hematocytometer. Unstained cells are spherical, refract light well, and are easy to visualize and count.

differentiation conditions for between 24 to 72 h. Flow cytometric analysis has shown that, in contrast to our previous +/−*ts*TA model of conditionally immortalized cortical cells (78), neuroepithelial cells differentiated by mitogen-withdrawal and extra-cellular matrix addition do not undergo a significant change in apoptosis as a function of differentiation state (80). Neuroepithelial cells undergoing differentiation in this model system also exhibit significant changes in their profile of secreted cytokines. Vascular endothelial growth factor (VEGF)-A and monocyte chemotactic protein (MCP)-1 are significantly decreased as neuroepithelial cells differentiate, whereas levels of granulocyte-macrophage colony stimulating factor (GM-CSF) increase (80). Furthermore, we have been able to use this model to show that ethanol exposure during the proliferation phase has a persistent (organizational) effect on the secreted cytokine profile during the neuronal differentiation period. Therefore, this model facilitates the temporal separation of major second-trimester developmental programs, that is, neuronognesis, migration and neuronal differentiation, which normally occur together in the developing brain. The advantage of this simplified model is that we can now expose neuroepithelial cells to a teratogen during the progression of one developmental program, and study the impact of that teratogen on subsequent developmental programs, thereby teasing apart the activational and organizational effects of teratogens.

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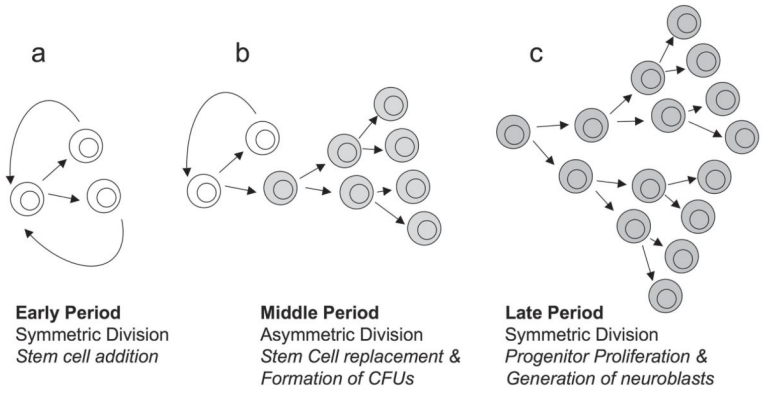

Fig. 1.

Schematic for modes of cell division observed in the stem cell beds. Symmetrical division **(a)** results in the generation of two daughter stem cells and permits the early expansion of the stem cell pool. Asymmetric division **(b)** in contrast, results in the generation of one stem and one more mature, progenitor daughter cell (e.g., in bone marrow, this results in the generation of a common lymphoid or myeloid progenitor, and a replacement stem cell). Asymmetric cell division therefore results in the maintenance of the stem cell pool. Subsequent cell divisions result in the clonal expansion of the progenitor pool (e.g., the transformation of a common myeloid progenitor to erythroid or myeloid CFUs). During the late period of maturation **(c)** symmetric cell division results in two daughter cells that are more mature, blast-type cells, resulting in a depletion of the parent progenitor pool (e.g., transformation of an erythroid CFU to a proerythroblast, resulting finally in the formation of an erythrocyte)

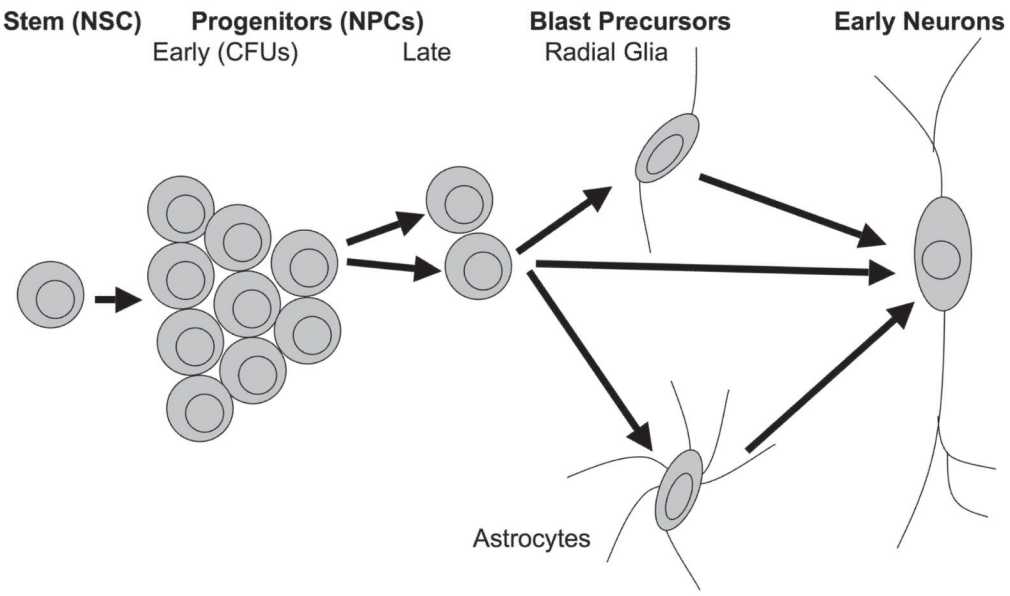

Fig. 2.

Presumptive maturation program of neural stem cells. Neural stem cells give rise to progenitors, which give rise to blast-type cells (radial glia and astrocyte-type cells), which can give rise to neurons

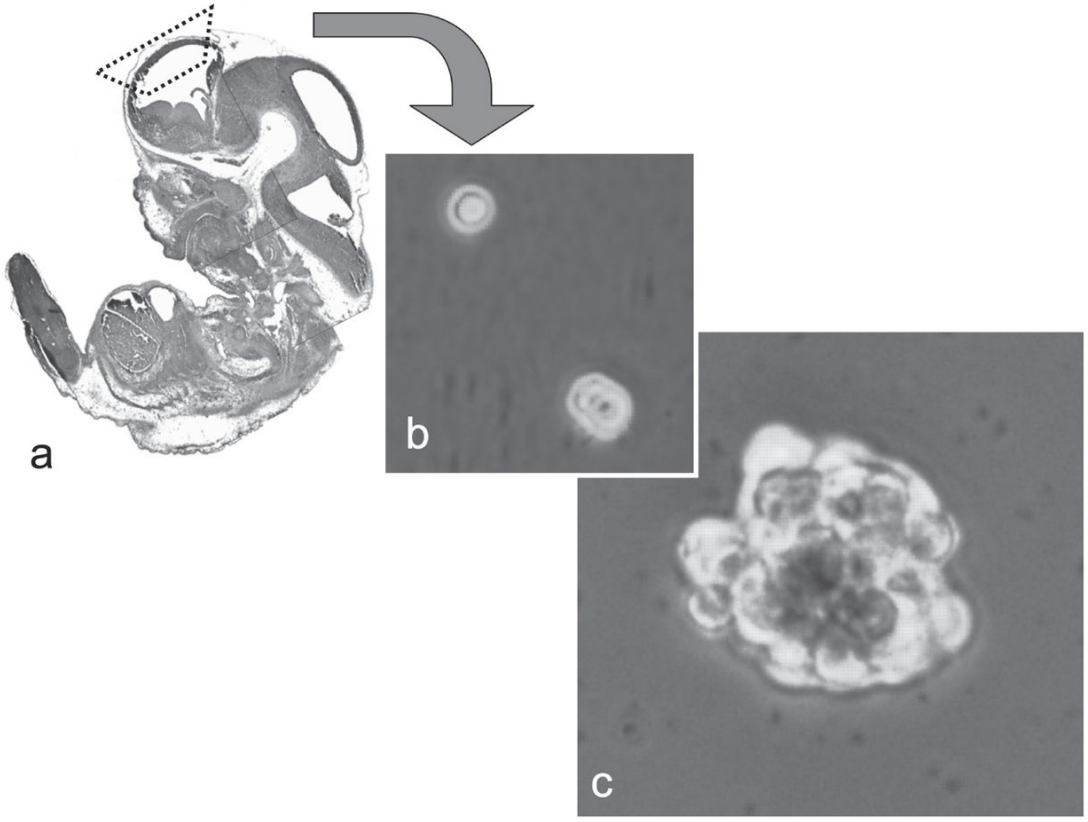

Fig. 3.

(a) Boxed area indicates the region from which neuroepithelial cells are isolated. (**b** and **c**) Neuroepithelial cells dispersed by trituration, and cultured in defined medium, generate nonadherent aggregates of cells referred to as neurospheres. The cellular composition of neurospheres is heterogeneous, and includes cells that express stem and progenitor markers (ABCG2, Sca-1, c-kit/CD117, CD133), nonselective markers of immature cells (nestin), markers for blast cells (Glial Fibrillary Acidic Protein, GFAP), as well as markers for early neuronal maturation (MAP-2). However, neurosphere cells do not express the neuron-specific marker, NeuN

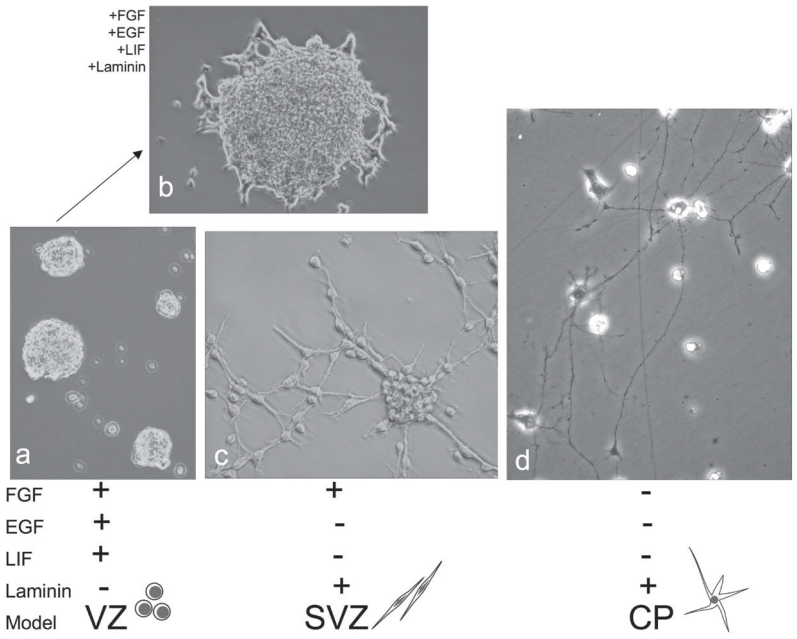

Fig. 4.

Schematic of the Mitogen-withdrawal, extra-cellular matrix addition paradigm. **(a)** The mitogenic condition results in an expansion of the nonadherent neurosphere population. Exposure to laminin in addition to mitogenic medium **(b)** results in neurospheres becoming adherent. However, migratory neuroepithelial cells retain an immature squamous epithelioid appearance. Withdrawal of EGF and LIF, and provision of laminin as an adhesion matrix **(c)**, results in neurospheres becoming adherent to the culture dish, and the appearance of bipolar migratory cells. These cells express nuclear NeuN, but do not exhibit nestin immunoreactivity, showing that these cells have transformed into migratory neurons. The additional removal of FGF (d) results in the appearance of multipolar neurons between 24 and 72 h

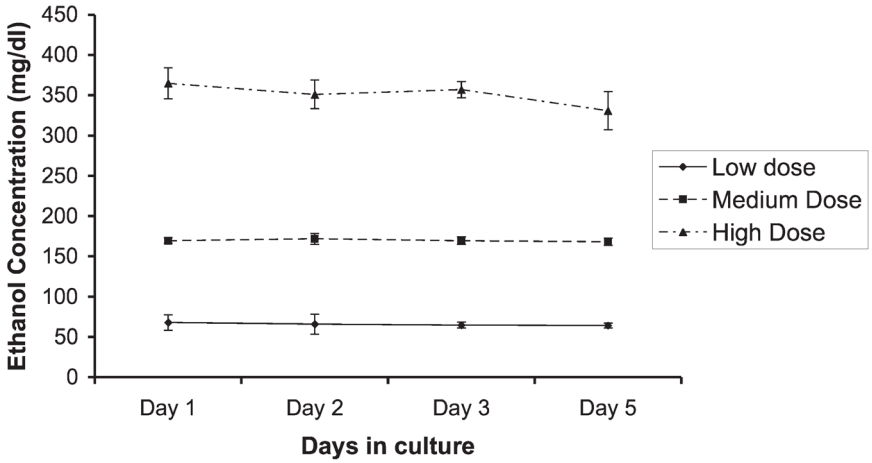

Fig. 5.

Gas chromatographic analysis of ethanol content in culture medium over a 5-d exposure period. Culture medium is replaced on day 3. The data show that ethanol concentrations remain stable over the period of the experiment