European Journal of Neuroscience, Vol. 24, pp. 3475–3488, 2006 doi:10.1111/j.1460-9568.2006.05239.x

Estradiol stimulates progenitor cell division in the ventricular and subventricular zones of the embryonic neocortex

Verónica Martínez-Cerdeño, Stephen C. Noctor and Arnold R. Kriegstein
Department of Neurology and Program in Developmental and Stem Cell Biology, 513 Parnassus Avenue, HSW 1201, Box 0525, University of California San Francisco, San Francisco, California 94143, USA

Keywords: cerebral cortex, intermediate progenitor cells, mice, proliferation, radial glial cells

Abstract

Two distinct populations of cerebral cortical progenitor cells that generate neurons during embryogenesis have been identified: radial glial cells and intermediate progenitor cells. Despite advances in our understanding of progenitor cell populations, we know relatively little about factors that regulate their proliferative behaviour. 17-β-Estradiol (E2) is present in the adult and developing mammalian brain, and plays an important role in central nervous system processes such as neuronal differentiation, survival and plasticity. E2 also stimulates neurogenesis in the adult dentate gyrus. We examined the role of E2 during embryonic cortical neurogenesis through immunohistochemistry, *in situ* hybridization, functional enzyme assay, organotypic culture and *in utero* administration of estradiol-blocking agents in mice. We show that aromatase, the E2 synthesizing enzyme, is present in the embryonic neocortex, that estrogen receptor-α is present in progenitor cells during cortical neurogenesis, that *in vitro* E2 administration rapidly promotes proliferation, and that *in utero* blockade of estrogen receptors decreases proliferation of embryonic cortical progenitor cells. Furthermore, the E2 inhibitor α-fetoprotein is expressed at high levels by radial glial cells but at lower levels by intermediate progenitor cells, suggesting that E2 differentially influences the proliferation of these cortical progenitor cell types. These findings demonstrate a new functional role for E2 as a proliferative agent during critical stages of cerebral cortex development.

Introduction

Recent work has identified two distinct populations of cerebral cortical progenitor cells that generate neurons during embryogenesis: radial glial cells in the ventricular zone (VZ; Malatesta *et al*., 2000; Miyata *et al*., 2001; Noctor *et al*., 2001; Tamamaki *et al*., 2001) and intermediate progenitor (IP) cells in the subventricular zone (SVZ; Tarabykin *et al*., 2001; Letinic *et al*., 2002; Haubensak *et al*., 2004; Miyata *et al*., 2004; Noctor *et al*., 2004; Englund *et al*., 2005). Despite recent advances in characterizing the properties of neuronal progenitor cells in the embryonic cerebral cortex, we know relatively little about the factors that regulate their proliferative behaviour.

17-β-Estradiol (E2) is present in the mammalian brain from embryonic stages of development through adulthood, and plays an important role in central nervous system processes such as neuronal differentiation, survival and plasticity (McEwen *et al*., 1991; Beyer, 1999; Toran-Allerand, 2004). In addition, E2 has been shown to act as a mitogenic factor (Clarke, 2004) and to stimulate neurogenesis in the dentate gyrus of adult rodent brain (Tanapat *et al*., 1999; Tanapat *et al*., 2005). Maternal E2, which is present in human serum at a concentration of 25 ng/mL (Troisi *et al*., 2003), crosses the placenta and the immature blood–brain barrier to reach the embryonic brain. α-Fetoprotein (AFP), which is present in the fetal blood, cerebrospinal fluid and cerebral extracellular matrix, binds E2 with high affinity and sequesters and inhibits the actions of maternal E2 (Adinolfi *et al*., 1976; Attardi & Ruoslahti, 1976; Adinolfi & Haddad, 1977; Soto & Sonnenschein, 1980; McCall *et al*., 1981; Mizejewski *et al*., 2004). Nevertheless, the E2-synthesizing enzyme aromatase is expressed in the cerebral cortex, which is consistent with local production of E2 in the cerebral cortex (MacLusky *et al*., 1986; Connolly *et al*., 1994). Two estrogen receptors (ER) that bind E2 have been described: ERα and ERβ. Both receptors are present in the cortex during postnatal development (Perez *et al*., 2003) and in neuronal stem cells purified from embryonic and adult rat (Brannvall *et al*., 2002). In addition, ERα has been detected in the embryonic cerebral cortex (Vito & Fox, 1979; Friedman *et al*., 1983)

E2 can have long-lasting genomic or classical actions as a transcription factor; these involve ligand binding to nuclear ERs and interactions with target gene promoters. E2 can also have rapid nongenomic or nonclassical actions by activating signal transduction molecules in postnatal cortical brain cells (Singh *et al*., 1999; Setalo *et al*., 2002; Setalo *et al*., 2005). This signaling is mediated via membrane-bound receptors that could be distinct from the classical nuclear ERs (Razandi *et al*., 2003). E2 has been shown to rapidly activate signaling cascades in noncortical brain areas by stimulation of mitogen-activated protein kinase (MAPK; Fatehi *et al*., 2005; Vasudevan *et al*., 2005). The intracellular signaling mechanisms of E2 have not been examined in the proliferative zones of embryonic cerebral cortex.

As E2 has been shown to stimulate neurogenesis in the adult brain (Banasr *et al*., 2001; Ormerod *et al*., 2004), we propose that it may regulate progenitor cell proliferation in the embryonic cerebral cortex.

Materials and methods

A total of 522 male and female, prenatal and postnatal, and two adult mice were used for this study. Animals were treated in accordance with protocols approved by the Institutional Animal Care and Use

Correspondence: Dr Verónica Martínez-Cerdeño, as above.
E-mail: vmc@ventricular.org

Received 4 September 2006, revised 12 October 2006, accepted 16 October 2006

Committee at UCSF. Mice were deeply anaesthetized using ketamine (90 mg/kg) and xylazine (7 mg/kg), and the embryos were removed and perfused or prepared for culture experiments.

Immunostaining

A total of 24 mice were used to prepared tissue for immunochemical studies. Early prenatal embryos, embryonic day (E)9–14, were fixed by immersion; older prenatal (E15–19), postnatal day (P)0–6, P13, P16 and adults were perfused intracardially with 0.1 M phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in PBS (PFA). Brains were removed, postfixed for 24 h in PFA at 4 °C, cryoprotected in 30% sucrose and embedded in agar (FisherBiotech, NJ, USA), and/or Tissue-Tek (Sakura, CA, USA). Tissue was frozen and coronal 30–40 μm slices were prepared on a cryostat (CM 3050S; Leica Microsystems, IL, USA). Free-floating sections were blocked in 10% donkey serum (Gibco, CA, USA), 0.1% Triton X-100 (Sigma, MO, USA) and 0.2% gelatin (Sigma). Sections were incubated for 24 h at room temperature in primary antibody. Sections were incubated in one of two antiaromatase antibodies. The first was a polyclonal aromatase antibody generated from a 12 amino acid peptide of mouse aromatase (amino acids 488–500; 1 : 500), a generous gift from Dr Hutchison and Dr Bicknell (Beyer *et al*., 1994; Hutchison *et al*., 1997; Garcia-Segura *et al*., 1999). We used a second polyclonal aromatase antibody raised against human placental aromatase (1 : 3000), a generous gift from Dr Harada (Harada, 1988; Jakab *et al*., 1993; Dellovade *et al*., 1995; Balthazart *et al*., 1996). Sections were incubated in one of three anti-ERα antibodies. The first was a mouse monoclonal antibody against human ERα that detects both the steroid-occupied and -unoccupied forms of the ERα and does not cross-react with ERβ. By Western blot, this antibody detects a 67 kDa synthetic peptide representing human ERα (1 : 100, RP-310; Affinity Bioreagents, CO, USA). We also used a polyclonal ERα antibody raised against the carboxy terminus of mouse ER (amino acids 580–599) that does not cross-react with ERβ (1 : 500, MC-20, sc-542; Santa Cruz Biotechnology, CA, USA). The third was a polyclonal antibody against the last 15 amino acids of rat ERα, which detects a 66 kDa protein representing ERα and does not cross-react with ERβ (1 : 10000, C-1355; Upstate Cell Signaling Solutions, NY, USA). We used two polyclonal antibodies against ERβ. The first was raised against a peptide corresponding to the amino terminus sequence of ERβ (1 : 50; Santa Cruz Biotechnology) and the second was raised against a peptide mapping to the amino terminus of mouse ERβ. This antibody does not cross-react with ERα (1 : 2000; Zymed Laboratories Inc., CA, USA). We also used α-fetoprotein antibody (1 : 50, Clone 189502; R & D Systems, MN, USA), monoclonal RC2 (1 : 100; Developmental Studies Hybridoma Bank, IA, USA); antinestin (1 : 500; Chemicon, USA); antivimentin (1 : 50; Sigma); Tuj1 (1 : 1000; Covance, CA, USA); anti-MAP2 (1 : 200; Chemicon); anti-NeuN (1 : 1000; Chemicon); monoclonal anti-4A4 (1 : 1000; MBL, MA, USA); antiphosphohistone H3 (1 : 1000; Upstate); fluorescent conjugated monoclonal antibromodeoxyuridine (BrdU; 1 : 20; Molecular Probes, OR, USA) and p44/42 phospho-(p-)MAPK (Thr202/Tyr204, 1 : 1000; Cell Signaling). Sections were washed and incubated in the appropriate secondary antibody: Cy2-, Cy3- or Cy5-conjugated polyclonal antimouse, -goat or -rabbit antibodies (1 : 400; Jackson Laboratories, ME, USA). Double immunostaining was performed (aromatase–RC2, ERα–RC2, aromatase–ERα, aromatase−phosphorylated vimentin (4A4), aromatase–BrdU, AFP−4A4). Antibodies were diluted in incubation buffer containing 2% normal donkey serum, 0.02% Triton X-100 and 0.04% gelatin. The nuclear marker Syto-11 (1 : 1000; Molecular Probes) was used for cytoarchitectonic imaging. To unequivocally ascertain immunostaining in the cell dense VZ tissue, we performed cell suspension immunostaining controls for each of the aromatase and the ERα antibodies. We dissociated VZ tissue by incubation with activated papain at 37 °C (Worthington Biochemical Corporation, NJ, USA), followed by mechanical trituration and fixation with PFA. We then double-labeled cells with RC2–aromatase and RC2–ERα antibodies. We also stained the cells with antibodies directed against the progenitor cell markers nestin or phosphohistone H3, and one of the following neuronal markers: Tuj1, MAP2 or NeuN. We omitted the first antibody as a control for each immunostaining experiment. We also performed a preadsorption control for ERα immunostaining using human recombinant ERα protein (1 μg/mL; Affinity Bioreagents, CO, USA). All imaging was performed on an Olympus Fluoview confocal laser scanning microscope and analysis performed in Fluoview v.3.3 (Olympus, CA, USA). For colocalization studies, expression of protein was confirmed by examining single confocal optical sections. All figures demonstrating coexpression of proteins were constructed from single optical sections of confocal images.

Western blot

Cortical tissue from 36 E15 embryos was collected in two Western blot experiments. The tissue was homogenized and lysed in 1% SDS buffer plus EDTA-free protease inhibitor (Roche, CA, USA). The supernatant was loaded onto 12.5% polyacrylamide gels (Bio-Rad, CA, USA) and then electroblotted onto PVDF membranes after electrophoresis (Bio-Rad). We used 25 and 75 kDa protein markers (Bio-Rad). Membranes were immunostained with the anti-ERα antibody (1 : 200; Santa Cruz) and amplified with a biotinylated secondary antibody (1 : 200; Jackson Laboratories), avidin–biotin complex (Vectastain; Vector Laboratories, CA, USA) and developed with 3,3′-diaminobenzidine and hydrogen peroxide (Sigma).

In situ *hybridization*

E. coli bacteria including a pT7T3-Pac plasmid containing aromatase (accession number BX100391) or ERα (accession number AA024073) cDNA inserted between EcoRI and NotI (BMAP library; Invitrogen, CA, USA) were grown and plasmid isolated (Qiagen, CA, USA). We cut the plasmid with EcoRI and NotI restriction enzymes (Promega, WI, USA) and synthesized sense and antisense RNA conjugated with digoxigenine (probe-DIG; Promega). We treated 20 μm coronal slices of neocortex from two E15 mice with proteinase K. Slices were acetylated, permeabilized with Triton X-100, prehybridized with herring fish DNA and yeast RNA (Promega) and then hybridized overnight at 72 °C with 300 ng/mL antisense (experiment) or sense (control) probe-DIG. We labeled the probes with antidigoxigenine-PA antibody, and detected labeled probe with NBT/BCIP in NTMT (Roche).

Aromatase activity assay

Following the method of Thompson & Siiteri (1974), pregnant females were anaesthetized, E15 fetuses removed from the uterus, and cortical tissue dissected from the brains in oxygenated artificial cerebrospinal fluid (ACSF). The tissue was homogenized in 1 mL of PKDE (containing in mM: potassium phosphate, 10; KCL, 100; dithiothreitol, 10; sodium EDTA, 1) per 100 mg of tissue, and then incubated with 1-β-[^{3}H]-androstenedione and NADPH for 1 h at 37 °C. We used PKDE in place of tissue as a negative control and embryonic amygdala and intestine as positive controls, and we added

the aromatase blocker letrazole to test the specificity of the activity assay. After 1 h the tissue and incubation medium was spun down at 750 ***g*** for 15 min and the supernatant removed. Radioactivity in the supernatant was measured in Optifluor liquid scintillator cocktail (Packard, CA, USA) on a Packard scintillator at an efficiency of 30%. A total of 80 embryos were used in eight experiments.

In vitro *E2 treatment*

Organotypic slice cultures (247) were prepared from the cerebral cortex of 70 E15 embryos. Pregnant animals were anesthetized and decapitated. Embryos were removed and fetuses removed from the uterus and brains maintained in oxygenated ACSF (in mM: NaCl, 125; KCl, 2.5; $MgCl_2$, 1; $CaCl_2$, 2; $NaPO_4$, 1.25; $NaHCO_3$, 25; and glucose, 25; Sigma). Coronal slices were cut at 350 μm on a vibratome (Leica, USA) and placed on Millicell inserts in six-well culture plates in culture medium containing 25% fetal bovine serum, 25% Hanks's balanced salt solution, 46% BME, 1 × penicillin–streptomycin and 0.6% glucose (Gibco). The slices remained in culture for 3 days in the presence or absence of 100 nM E2 (Sigma) and the presence or absence of the ER blockers tamoxifen (40 nM; Sigma) or ICI (100 nM, ICI 182,780; Tocris, UK). In some experiments we added BrdU (5 μM) to the medium for the last hour of the culture period. After 3 days the slice cultures were fixed overnight with PFA, immunostained with the 4A4 antibody, counterstained with Syto-11 (Molecular Probes) and imaged with confocal microscopy. Dorsolateral cortex was imaged at 20× and all $4A4^+$ or $BrdU^+$ cells in the VZ and SVZ in a field 500 μm wide were counted, and the numbers of $4A4^+$ dividing radial glial cells were compared across groups as described below.

Some slices were prepared as described above, but were maintained for 3 h in oxygenated ACSF (266 slices, 93 embryos). The slices were maintained in the presence or absence of 100 nM E2 and the presence or absence of the ER blockers tamoxifen (40 nM) or ICI (100 nM). In some experiments we added BrdU (5 μM) to the mediim for the last hour of the culture period. The slices were fixed and immunostained, and the numbers of $4A4^+$ or $BrdU^+$ cells were quantified and statistical analysis performed as described below.

In vitro *androstenedione treatment*

Thirty-three slices were prepared from 12 embryos in three experiments as above and cultured in the presence of 5.0 or 0.5 nM androstenedione for 3 h. Slices were processed and analysed as described below.

In vitro *tamoxifen and ICI assays*

For tamoxifen, E15 pregnant females were injected intraperitoneally with tamoxifen (1.8 mL, 100 nM). Five hours later fetuses (12) were anesthetized through hypothermia removed and perfused with PFA. For ICI, which does not cross the placenta, we injected it *in utero* into the lateral ventricles of the fetuses. Uterine horns of E15 pregnant female were exposed and ICI (2 μL of 40 nM ICI) was injected using glass micropipettes. Uterine horns were replaced into the peritoneal cavity and the wound closed. Five hours later fetuses (12) were removed and perfused with PFA. Slices 100 μm thick were cut on a vibratome (Ted Pella, CA, USA) and immunostained with 4A4 as described above. Twelve fetuses from noninjected pregnant females were used as controls. The numbers of $4A4^+$ cells in the VZ and the SVZ were quantified and statistically compared as described below.

Intracellular signaling pathway determination

Cultured slices from 48 embryos were prepared as described above and treated with E2, or E2 and the extracellular signal-regulated kinase (ERK)1/2-specific blocker PD-98059 (10 μM; generous gift of Dr Paul Webb). After 1 h of treatment, slices were fixed and immunostained with phospho-p44/42 MAPK (Thr202/Tyr204; Cell Signaling) and 4A4 antibodies and imaged on a confocal microscope, and the numbers of p-ERK1/2-positive ($p\text{-}ERK^+$) and $4A4^+$ cells were quantified in each group.

Dose–response assay

E15 slices were prepared and maintained for 3 h in oxygenated ACSF as described above in the presence of E2 (nine concentrations ranging from 10 nM to 1000 μM). After 3 h the slices were fixed and immunostained with 4A4 antibody. The numbers of $4A4^+$ cells were quantified and compared across groups as described below. A total of 235 slices were prepared from 38 embryos in five dose–response E2 treatment experiments.

Sampling, quantification and statistics

To examine coexpression of proteins in immunostaining experiments we examined single optical sections prepared from coronal sections of the neocortex imaged through confocal microscopy (Olympus). Images of the dorsolateral cortex were taken along the rostrocaudal axis of the neocortex to account for any regional differences. Analysis was always performed in dorsolateral cortex of the sensorimotor area. The number of double-positive cells was counted in coronal slices prepared from each animal, and statistical significance tested on results from a minimum of three animals in each case.

To compare proliferation *in vitro*, cultured coronal sections obtained from the sensorimotor area of developing neocortex were immunostained as described above. The dorsolateral cortex of each slice was imaged at 20× on a confocal microscope (Olympus). Projection images for analysis were generated from optical sections taken at 3 μm steps within the immunostained portion of the slice. The numbers of $4A4^+$, $BrdU^+$ or $p\text{-}ERK^+$ cells in each condition were quantified in a 500-μm-wide bin that extended radially from the ventricular surface of the coronal slice and included the VZ and SVZ of dorsolateral cortex. A positive cell soma was counted as a positive cell. The total number of positive cells per area was normalized across groups for each experiment, and results statistically compared across experiments. Mann–Whitney and Student's *t*-tests were performed on the normalized values using Instat (GraphPad Software, CA, USA). The numbers of embryos and slices used for analysis are specified for each experiment in the results section.

PCR gender determination

Ninety-seven organotypic slice cultures from 24 E15 embryos in three separate experiments were prepared and treated with E2 as described above. DNA was extracted from the livers of each embryo as a lysate through proteinase K digestion. Approximately one milligram of the liver was excised and placed in 400 mL of 200 mg/mL proteinase K (50 mM Tris-Cl, 100 mM EDTA and 0.5% SDS, pH 8.0). The samples were incubated at 50 °C overnight before high-salt protein precipitation (5 M NaCl). The DNA was precipitated with 300 mL isopropanol and pelleted by centrifugation. After a brief 70% ethanol wash and air-dry, the pellets were dissolved in 50 mL Tris-EDTA (pH 8.0) buffer. DNA (1 ml) was

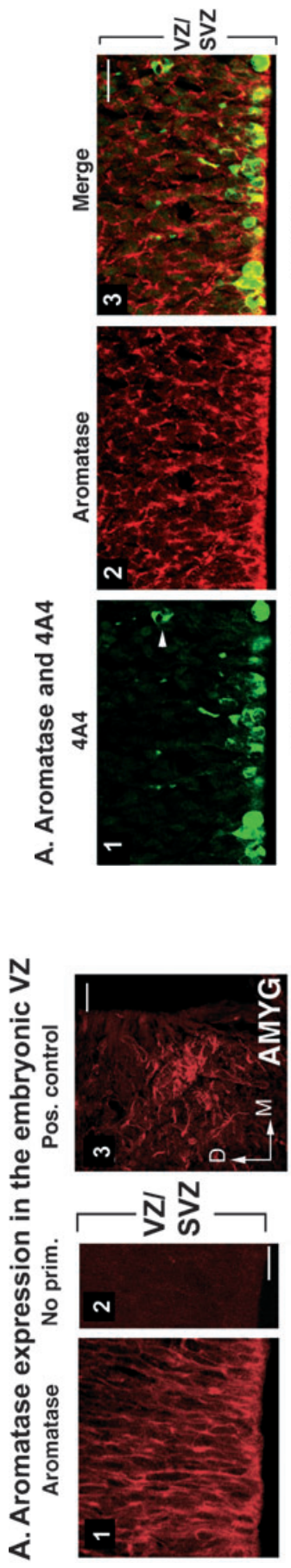

FIG. 1. Radial glial cells expressed aromatase in the cortical VZ. (A1) Anti-aromatase antibody strongly labels E15 mouse VZ and SVZ. (A2) Negative control experiments in which the primary antibody was omitted demonstrate a lack of immunostaining. (A3) Positive control experiments show aromatase$^+$ cells in the amygdala. Dorsal and medial are indicated with arrows. (B1–3) Co-labeling with the radial glial marker RC2 (green) shows that most radial glial cells in the cortical VZ express aromatase (red). (B4–7) Single-cell suspension prepared from the embryonic VZ at E15 shows RC2 (green) and aromatase (red) colabeling. (B8–11) Morphologically identified radial glial cell labeled with eGFP-expressing retrovirus (green) demonstrates expression of aromatase (red) in the perinuclear soma (white arrowheads). Aromatase messenger RNA is present in the cortical proliferative zones. (C1) Aromatase antisense probe densely labels the VZ, especially at the ventricular surface. (C2) Sense control probe did not produce any label. Scale bars, 20 μm (A1 and 2), 25 μm (A3; in B7 for B4–7), 10 μm (B1–3), 15 μm (B8), 5 μm (B9–11), 50 μm (C1 and 2).

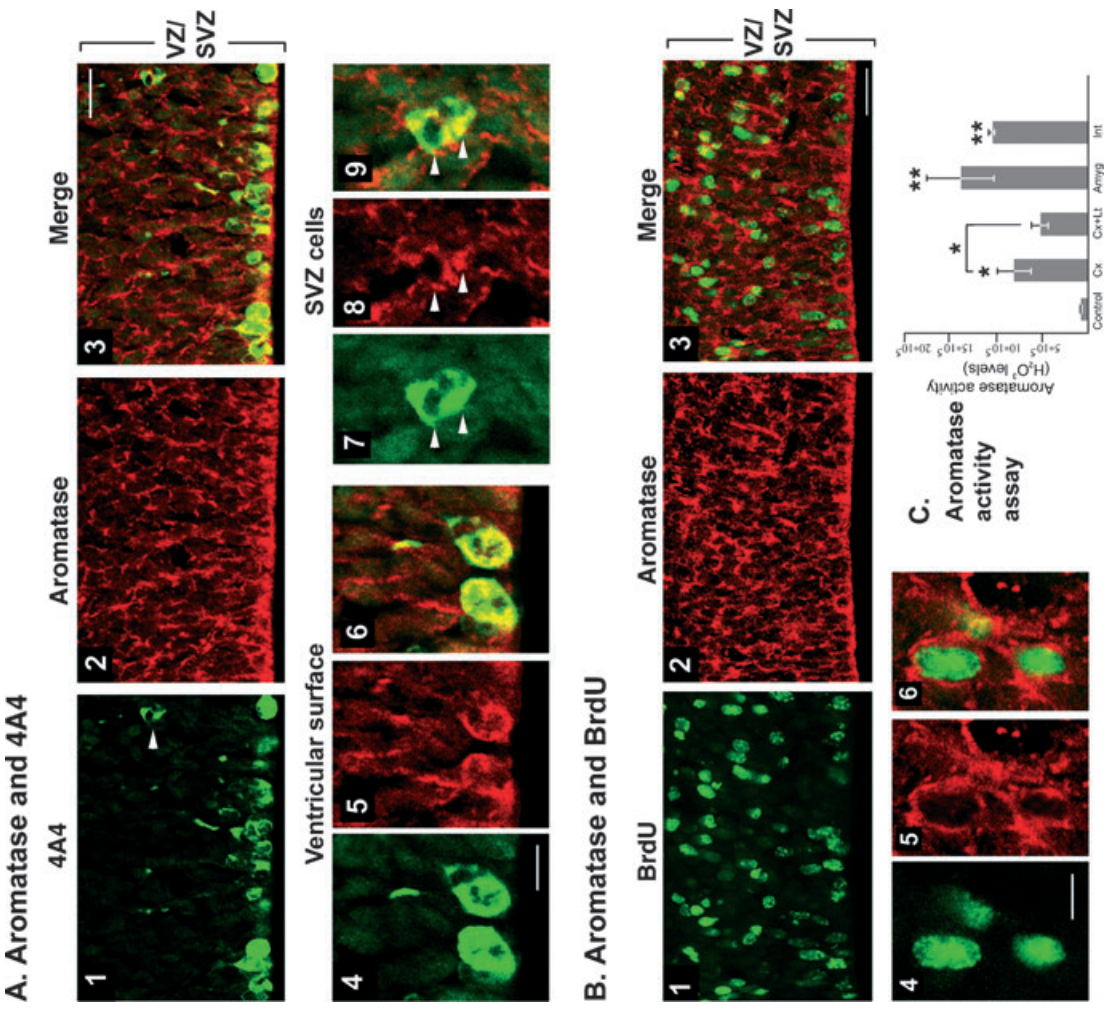

FIG. 2. Mitotically active radial glial cells expressed aromatase. (A1) 4A4 staining in the VZ. White arrowhead indicates a 4A4$^+$ cell in the SVZ. (A2) Aromatase staining in the VZ. (A3) Merged image demonstrates colabeling. (A4–6) Higher power magnification shows 4A4$^+$ cells (green) that also express aromatase (red). (A7–9) Higher power magnification of a SVZ cell that coexpresses 4A4 (green) and aromatase (red). (B1–3) S-phase BrdU$^+$ cells (green) coexpress aromatase (red) in the E15 cortical VZ. (B4–6) Higher power images show BrdU$^+$ cells (green) that express aromatase (red) in the VZ. (C) Histogram showing aromatase activity assay in E15 mouse. Neocortex shows significant aromatase activity above background. Activity was significantly reduced when the aromatase blocker letrazole (Ctx + Lt) was added to the incubation medium. Fetal amygdala and intestine also showed high levels of aromatase activity. Scale bars, 15 μm (A1–3), 5 μm (in A4 for A4–9 and B4 for B4–6), 25 μm (B1–3). $^*P < 0.05$, $^{**}P < 0.01$.

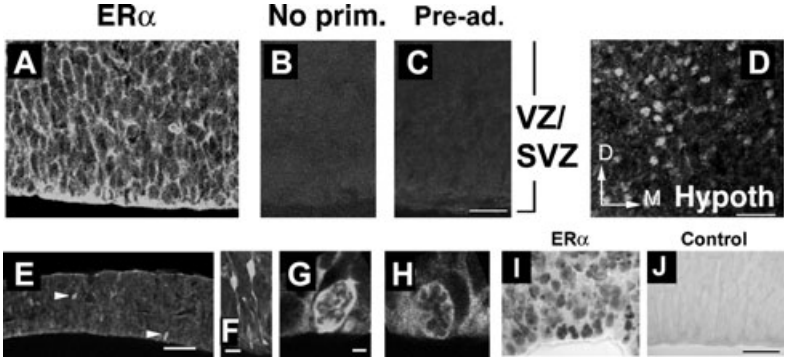

FIG. 3. ERα was expressed in the VZ and SVZ of the developing cerebral cortex. (A) ERα expression in coronal sections of E15 mice stained with antibodies raised against ERα. (B) Negative control experiments in which the primary antibody was omitted. (C) Preadsorption of the primary antibody with ERα protein abolished immunostaining. (D) Positive control experiments show that the antibody labels ERα$^+$ cells known to be present in the developing hypothalamus. Dorsal and medial are indicated with arrows. (E) Anti-ERα immunostaining in the E12 mouse cortex shows that only a few cells in the VZ expressed ERα. (F–H) By E16, ERα immunostaining is present in VZ cells that have the morphology of radial glial cells, and (G and H) that appear to be in the M-phase at the edge of the ventricle. (I) ERα antisense probe also labels the VZ strongly. (J) ERα sense probes do not produce label. Scale bars, 25 μm (in C for A–C; E), 30 μm (D, and in J for J and I), 5 μm (F), 2 μm (G and H).

added to Supermix (Invitrogen Life Technologies) for PCR amplification using two sets of primers (Invitrogen Life Technologies): male-specific Sry primers (5′-TCATGAGACTGCCAACCACAG-3′, 5′-CATGACCACCACCACCACCAA-3′) and Myog-specific primers (5′-TTACGTCCATCGTGGACAGC-3′, 5′-TGGGCTGGGTGTTAG-TCTTA-3′). A thermal cycler (Thermolyne Amplitron I) was used with the following programming conditions: one cycle at 94 °C, 8 min; 30 cycles at 94 °C, 45 s; 55 °C, 30 s; and 72 °C, 1.30 min; and one cycle at 72 °C, 10 min. PCR products were separated on a 1.2% agarose gel through electrophoresis for 30 min at 120 V and visualized with ethidium bromide under UV illumination. A total of 97 slices were prepared from 24 embryos in three PCR experiments.

Apoptosis assay

To determine whether the increase in mitosis was due to an inhibition of cell death among VZ cells, we quantified the number of apoptotic cells in the VZ after the culture period. One hundred and forty-five slices were prepared from 39 E15 embryos in four experiments and cultured in E2 or control medium for 3, 12 or 24 h or 3 days as described above. The TUNEL assay (Roche) was performed as per instructions. We performed TUNEL-positive and -negative controls. To obtain the TUNEL-positive control we treated the slices with DNase I. Slices were imaged with confocal microscopy (Olympus), and the number of cells quantified and compared statistically as described above.

Virus production and animal surgeries

Replication-incompetent enhanced green fluorescent protein (eGFP)-expressing retrovirus was produced from a stably transfected packaging cell line (293 gp NIT-GFP; gift of Dr Fred Gage). Cells were transiently transfected at 80% confluence with pVSV-G using the Calphos Mammalian Transfection kit (Clontech, CA, USA). Supernatant was harvested 48 h after transfection, filtered through 0.45 μm low-protein-binding filters (Fisher Scientific) and concentrated at 25 000 ***g***, 4 °C for 1.5 h. Pellets were resuspended in Opti-MEM (Invitrogen) and stored at 80 °C. Uterine horns of E15 timed pregnant rodents were exposed in a sterile biosafety level II hood. Retrovirus (∼ 0.5 μL) with Fast Green (2.5 mg/mL, Sigma) was injected into the cerebral ventricles through a beveled calibrated glass micropipette (Drummond Scientific, PA, USA). After injection, the peritoneal cavity was lavaged with 10 mL of 0.9% NaCl, the uterine horns were replaced and the wounds were closed. After 24 h fetuses were removed and perfused with PFA. Brains were frozen, sectioned on a cryostat, immunostained and imaged on a confocal microscope as described above.

Results

Aromatase is expressed by radial glial cells and IP cells

We examined the expression pattern of aromatase in the developing neocortex using two antibodies directed against aromatase protein (Supplementary material, Fig. S1). Each antibody detected aromatase immunoreactivity in regions of the developing and adult central nervous system known to express aromatase, such as the hypothalamus and the amygdala (Fig. 1, A3; Balthazart *et al*., 1990; Balthazart *et al*., 1991; Foidart *et al*., 1995; Sasano *et al*., 1998). We also found that aromatase was expressed in the embryonic VZ and SVZ starting on E9. We observed strong aromatase expression in the VZ throughout the remainder of embryonic development and in the early postnatal period. Aromatase expression was particularly intense at the ventricular surface where radial glial cells divide (Fig. 1, A1). We tested whether radial glial cells express aromatase by double-labeling tissue with antirat aromatase antibody and the radial glial-specific marker RC2. At E15 we found that 89 ± 0.05% of aromatase$^+$ cells also expressed RC2 (n = 536 cells, five embryos), and noted that radial glial cells expressed aromatase in the cytoplasm, the ventricular processes and the pial-directed radial fibers (Fig. 1, B1–3). VZ cells are packed close together; we therefore prepared single-cell suspensions from the VZ of eight embryos, performed the same double-labeling assays and found that 99 ± 0.01% of the single cells expressed both markers (Fig. 1, B4–7). A small percentage of the single cells (< 10%) stained positive for the immature neuronal marker Tuj1, and none stained positive for the mature neuronal markers MAP2 or NeuN. To further confirm the cell-specific expression of aromatase, we labeled embryonic progenitor cells with *in utero* injections of an eGFP-expressing retrovirus, a method that labels radial glial cells (Noctor *et al*., 2001, 2004). Twenty-four hours after retroviral injection embryos were killed, and sectioned tissue was costained with aromatase antibodies. Single confocal optical sections showed that eGFP-labeled radial glial cells (Fig. 1, B8) expressed aromatase (Fig. 1, B9–11). We also tested for the presence of aromatase messenger RNA in the embryonic cortex and found that antisense probes produced strong labeling in the cortical VZ and SVZ (Fig. 1, C1). The *in situ* probes

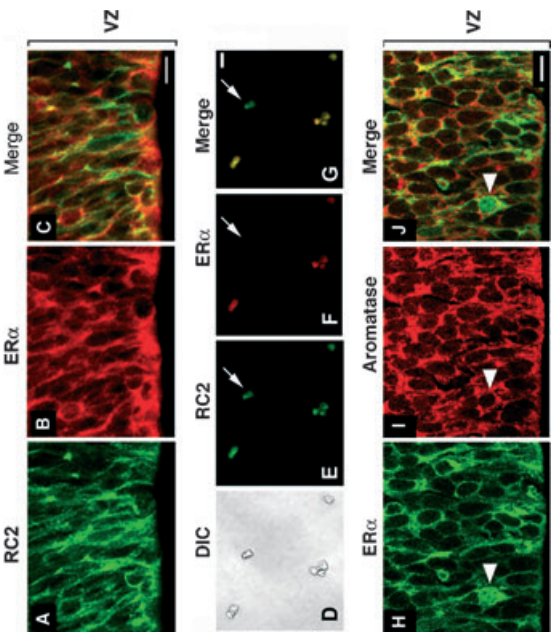

FIG. 4.

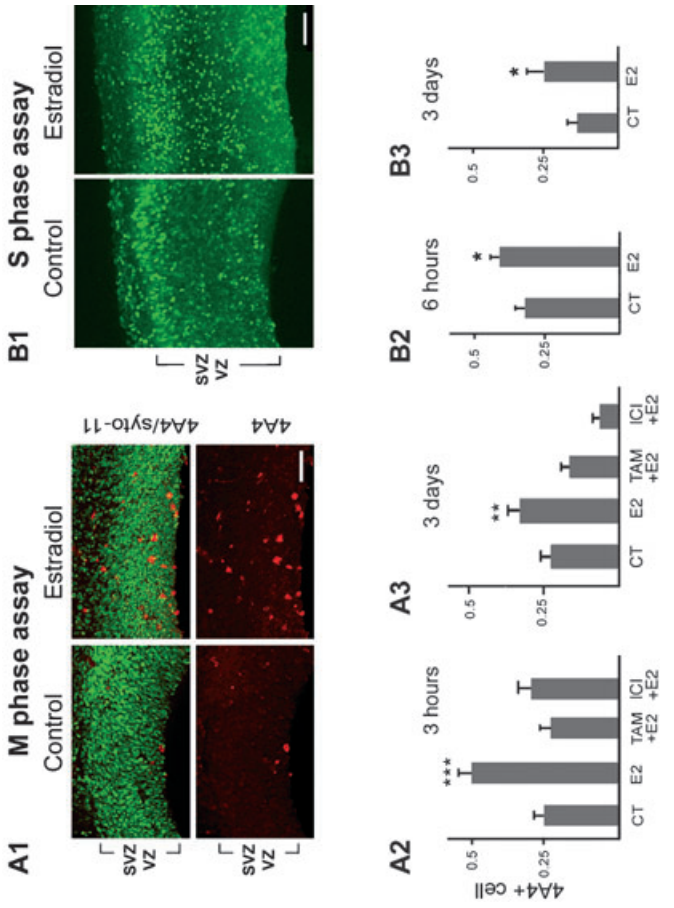

FIG. 6.

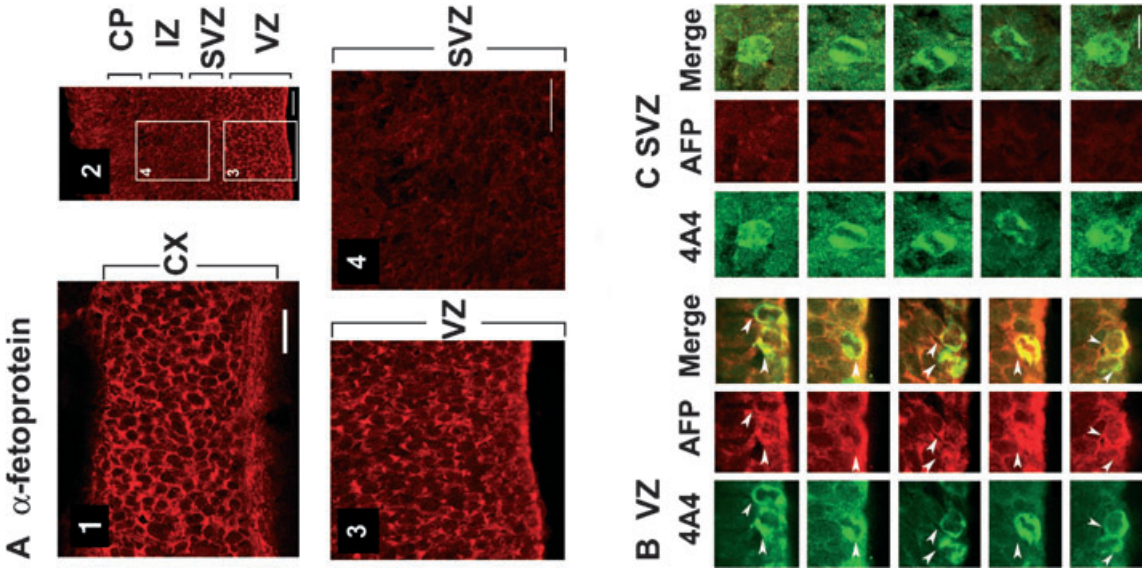

FIG. 5.

produced the typical pattern of staining that is observed in embryonic ventricular zone cells, which are very small and have very little cytoplasm surrounding the nucleus (Osman *et al*., 2004).

It has been reported that not all radial glial cells are mitotic during cortical development (Schmechel & Rakic, 1979). We therefore examined whether actively dividing radial glial cells express aromatase. We injected pregnant mice at E15 with a single dose of BrdU to label S-phase cells, and killed embryos 1 h later. Neocortical slices were labeled with antiaromatase antibody and costained with either an antibody directed against phosphorylated vimentin (4A4), a marker of M-phase radial glial cells (Kamei *et al*., 1998), or with anti-BrdU antibody. We found that most $4A4^+$ cells also expressed aromatase (95%; $n = 57$ cells; Fig. 2A), and that all $BrdU^+$ cells in the VZ also expressed aromatase ($n = 205$ cells, three embryos; Fig. 2B), confirming that mitotically active radial glial cells express aromatase. IP cells in the embryonic SVZ have recently been shown to generate neurons (Tarabykin *et al*., 2001; Letinic *et al*., 2002; Haubensak *et al*., 2004; Miyata *et al*., 2004; Noctor *et al*., 2004; Englund *et al*., 2005), and IP cells can also be labeled with 4A4 antibody (Noctor *et al*., 2004). We found that the majority of $4A4^+$ IP cells in the SVZ also coexpressed aromatase (Fig. 2, A7–9).

We next tested, using a radioactivity enzyme assay (Thompson & Siiteri, 1974), whether the aromatase enzyme detected with antibodies represented active enzyme capable of synthesizing E2. We found that E15 cortical tissue produced levels of $^3[H]_2O$ that were 10× above background ($P < 0.01$, $n = 8$ experiments), and that addition of the aromatase enzyme blocker, letrazole, to the tissue significantly reduced the levels of radioactivity by 37% ($P < 0.01$, Fig. 2C). We included tissue from the embryonic amygdala and intestine, organs known to have aromatase activity, as positive controls and noted that they produced significantly higher levels of radioactivity than cortical tissue (Fig. 2C). These experiments indicate that, in the presence of substrate, aromatase in the embryonic cortex may provide a local source of E2 during embryonic cortical development.

ERα is expressed by radial glial cells in the embryonic neocortex

The presence of ERs in the embryonic cortex has previously been reported (Vito & Fox, 1979; Friedman *et al*., 1983), but ER expression in the proliferative zones of the embryonic cerebral cortex has not been studied in detail. We examined the regional and cellular distribution of ERα and ERβ in the embryonic neocortex using three distinct antibodies directed against ERα and two distinct antibodies directed against ERβ (supplementary Fig. S2). We did not detect ERβ in the developing neocortex but did detect strong ERβ immunoreactivity in the adult cerebral cortex as previously reported (Mitra *et al*., 2003). In contrast, we found that each of the ERα antibodies labeled numerous regions of the embryonic brain, including cell nuclei in the hypothalamus as previously described (Fig. 3D; Mitra *et al*., 2003). In addition, each of the ERα antibodies produced strong immunolabeling in the VZ of the embryonic neocortex (Fig. 3A). Omission of the primary antibodies (Fig. 3B) or preadsorption of the primary antibodies with ERα protein (Fig. 3C) abolished the immunostaining.

We observed temporal changes in the pattern of ERα expression in the developing cerebral cortex. ERα immunostaining was not detected in the VZ at early stages of cortical development, from E9 to E11, but became apparent between E12 and E14 (Fig. 3E). Many labeled cells had bipolar radial glial cell morphology (Fig. 3F), and other cells appeared to be in the M-phase at the surface of the lateral ventricle where radial glial cells divide (Fig. 3G and H). Expression of ERα was stronger in the cortical VZ at E15–16 (Fig. 3A), and began to decrease in most areas of the cortex at E17–18, except in the cingulate cortex. Finally, we tested for the presence of ERα mRNA in the E15 cortical VZ and found that antisense probes produced strong label in the cortical VZ throughout the telencephalon (Fig. 3I).

To determine whether $ER\alpha^+$ cells with radial glial cell morphology were indeed radial glia, we colabeled E15 tissue with the antihuman ERα antibody and the radial glial marker RC2. We found that the vast majority (99 ± 0.03%; 386 cells, $n = 6$ embryos) of $ER\alpha^+$ cells in the VZ also expressed RC2 (Fig. 4A–C). ERα expression was localized to the soma, ventricular process, pial fibre and sometimes to the nucleus of $RC2^+$ radial glial cells. As VZ cells are densely packed we prepared single-cell suspensions from freshly fixed E15 cortex, and costained the cells with ERα and RC2 antibodies. We found that the vast majority of the $ER\alpha^+$ cells were also colabeled with RC2 (Fig. 4D–G) but did not stain positive for the mature neuronal markers NeuN or MAP2. Finally, as aromatase and ERα were both highly expressed by embryonic VZ cells, we colabeled E15 tissue with aromatase and ERα antibodies and found that these proteins were coexpressed by the majority of VZ cells (96 ± 0.03%; 1116 cells, $n = 6$ embryos; Fig. 4H–J).

ERα was originally described as a nuclear receptor, but has also been shown in the cytoplasm (Pietras & Szego, 1977; Parikh *et al*., 1987; Blaustein & Turcotte, 1989; Blaustein, 1992; Lehman *et al*., 1993; Milner *et al*., 2001; Zsarnovszky & Belcher, 2001; Razandi *et al*., 2003). We detected the presence of both nuclear and cytoplasmic ERα in embryonic VZ cells. The majority of VZ cells expressed the ERα in the cytoplasm and membrane only (80 ± 3%; 680 cells, $n = 5$ animals), while 20 ± 3% expressed ERα in both the nucleus and the cytoplasm and membrane (Fig. 4H, arrowhead). In contrast, the expression pattern was nearly reversed in the embryonic cortical plate where immature neurons differentiate; 31.5 ± 0.03% of cortical plate cells expressed ERα only in the cytoplasm and membrane, while 68.5 ± 0.03% expressed ERα in both the nucleus and the cytoplasm and membrane ($n = 490$ cells; supplementary Fig. S3).

FIG. 4. Radial glial cells in the VZ express the estrogen receptor ERα. (A–C) Expression patterns of the radial glial marker RC2 (green) and of ERα (red) in the E15 mouse. Merged image shows colabeling (yellow). (D–G) Differential interference contrast image shows single-cell suspensions prepared from the cortical VZ at E15 labeled with RC2 (green) and ERα (red). Merged image shows colabeling (yellow). White arrow points to an $RC2^+$ cell that does not express ERα. (H–J) ERα (green) and aromatase (red) expression in the VZ; merged image shows colabeling (yellow). Scale bars, 10 μm (in C for A–C and in J for H–J), 25 μm (in G for D–G).

FIG. 5. AFP was expressed at high levels by radial glial cells but not by IP cells. (A1) At E12, AFP was present throughout the developing neocortex. (A2) At E15, AFP was present in the VZ and also the cortical plate (CP), but was expressed at much lower levels in the SVZ. (A3 and 4) Higher magnification of insets in panel A2 show high expression of AFP in the VZ but much lower levels of expression in the SVZ. (B) $4A4^+$ radial glial cells in the VZ (green) coexpress AFP (red). (C) However, $4A4^+$ IP cells in the SVZ (green) coexpress little or no AFP (red). IZ, Intermediate zone. Scale bars, 40 μm (A1), 100 μm (A2), 50 μm (in A4 for A3 and 4), 5 μm (in C for C and B).

FIG. 6. E2 treatment increased the number of $4A4^+$ progenitor cell mitoses in the proliferative VZ and SVZ. (A1) The number of $4A4^+$ cells (red) in the VZ and SVZ was significantly increased after E2 treatment. Syto-11 staining (green). (A2 and 3) Histograms depicting the number of mitotic radial glial cells in the proliferative zones after E2 treatment for 3 h or 3 days. There was a significant increase in the number of radial glial mitotic figures after E2 treatment. Application of the E2 receptor blockers tamoxifen (TAM) or ICI abolished the increase in mitoses after E2 treatment after both 3 h and 3 days. E2 treatment increased the number of S-phase cells in VZ and SVZ E15 organotypic cultures. (B1) Anti-BrdU immunostaining is shown in control and treated slices after E2 treatment. (B2 and 3) Histogram showing a significant increase in the number of $BrdU^+$ cells in the VZ and SVZ after 6 h or 3 days E2 treatment. Scale bars, 50 μm. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

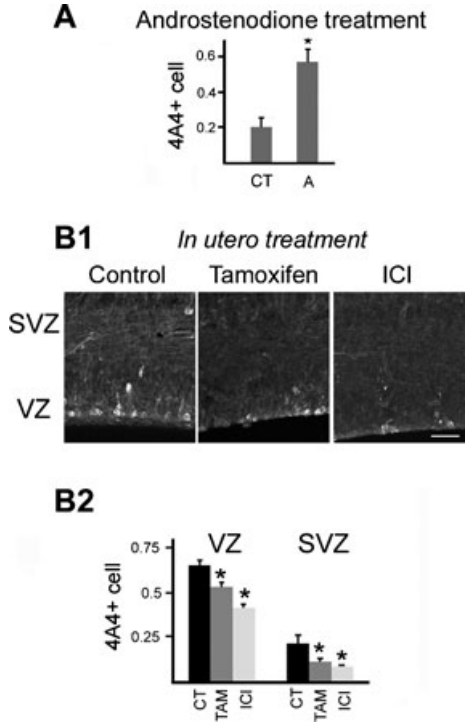

FIG. 7. ER blockers inhibited proliferation promoted by E2 *in vivo*. (A) Treatment of slices with androstenedione (A) for 3 h produced a significant increase over control (CT) in the number of $4A4^+$ mitoses. (B1) *In utero* treatment with the ER blockers tamoxifen or ICI for 5 h reduced proliferation in the E15 neocortex. In control embryos (left panel), mitotic $4A4^+$ cells in the VZ line the ventricular surface, while in the tamoxifen-treated (centre panel) and ICI-treated (right panel) embryos there is a significant reduction in the number of $4A4^+$ cells. (B2) Histogram showing that the number of $4A4^+$ progenitor cells in both the VZ and SVZ decreased significantly 5 h after treatment with tamoxifen (TAM) or ICI. Scale bar, 50 μm. $*P < 0.05$.

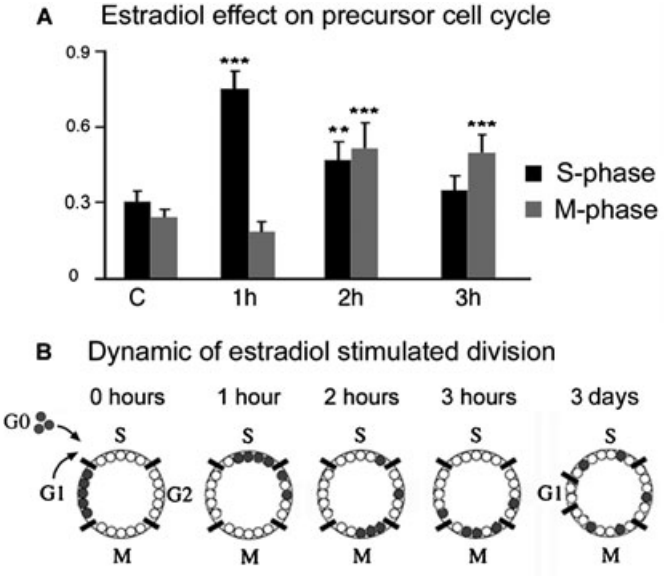

FIG. 8. E2 stimulated progenitor cells to enter the cell cycle. (A) Histogram showing that after 1 h of E2 treatment the number of cells undergoing S-phase ($BrdU^+$, black bars) was significantly increased while the number of M-phase cells ($4A4^+$, grey bars) was not changed. After 2 h of E2 treatment, the number of S-phase cells had decreased slightly but was still significantly higher than control, and the number of M-phase cells had increased above controls. After 3 h of treatment, the number of S-phase cells was similar to control while the number of M-phase cells remained elevated. (B) Scheme showing that E2 promotes cortical progenitor cells to enter the S-phase from either G1- or G0-phase after 1 h of E2 treatment. These cells progress through the G2-phase and are detected in the M-phase after 2 h of E2 treatment. E2 also reduces the length of the G1-phase, increasing the number of both S-phase and M-phase cells after 3 days E2 treatment. $**P < 0.01$, $***P < 0.001$.

Recently, a novel ER called ER-X has been described in adult brain (Toran-Allerand *et al*., 2002). ER-X expression is enriched in caveolar-like microdomains of plasma membranes and appears to be recognized by antibodies directed against ERα (Toran-Allerand *et al*., 2002). To test whether our antibodies might detect ER-X, we dissociated embryonic neocortical proteins on an agarose gel and studied the specificity of ERα antibodies through Western blotting. We found that the ERα antibodies reacted strongly with a 67 kDa band that presumably represents ERα, and more faintly with a 62–63 kDa band that may represent ER-X (data not shown).

AFP is expressed by radial glial cells but not by IP cells

AFP has been detected in the embryonic brain (Attardi & Ruoslahti, 1976), even at stages before the cerebral vasculature has developed, suggesting that this protein plays an important functional role during cortical development (Reynolds *et al*., 1983). We examined the distribution of AFP in neocortical tissue prepared from E12, E15 and E18 embryos with an antibody directed against human AFP. We found that AFP was strongly expressed in the E12 cortex, a stage of development when the cortex consists primarily of the VZ (Fig. 5, A1). At E15, when the SVZ has appeared in the developing cerebral cortex, we found strong AFP expression in the VZ but markedly lower levels of expression in the SVZ (Fig. 5, A2–4). We observed the same pattern of AFP expression at E18, when the SVZ is more fully developed. As AFP immunostaining in the VZ was stronger than in the SVZ, we compared AFP expression by radial glial cells in the VZ and IP cells in the SVZ. Sections of E15 neocortex were stained with 4A4 antibody, which labels mitotic radial glial cells at the VZ surface (Kamei *et al*., 1998) and mitotic IP cells in the SVZ (Noctor *et al*., 2004). We found that all $4A4^+$ radial glial cells dividing at the ventricular surface stained positive for AFP (Fig. 5B). In contrast, $4A4^+$ IP cells dividing in the SVZ expressed very little or no AFP (Fig. 5C).

E2 increased progenitor cell proliferation in the embryonic cerebral cortex

We found that ERα was expressed in the embryonic cortical proliferative zones and, as it is also expressed in neurogenic regions of the adult brain and it has been reported that E2 promotes neurogenesis in the adult brain (Tanapat *et al*., 1999, 2005), we tested whether E2 promotes progenitor cell division in the embryonic neocortex by treating organotypic cortical slice cultures with 100 nM E2. The concentration was chosen based upon dose–response experiments using organotypic cortical slice cultures treated with E2 (10 nM to 100 μM) for 3 h. Slices were fixed and immunostained with 4A4 antibodies. 4A4 antibodies label dividing M-phase cells in the embryonic cerebral cortex; these include radial glial cells and IP cells. These two populations of progenitor cells can be distinguished because they go through the M-phase in different locations. Radial glial cells undergo division at the ventricular surface while IP cells divide away from the ventricular surface, in the subventricular zone. In dose–response experiments we found that the rate of $4A4^+$ progenitor cell division was modulated by E2, with a peak of proliferation occurring between 25 and 100 nM. In addition, the dose we used falls within the range of concentrations that activate the known

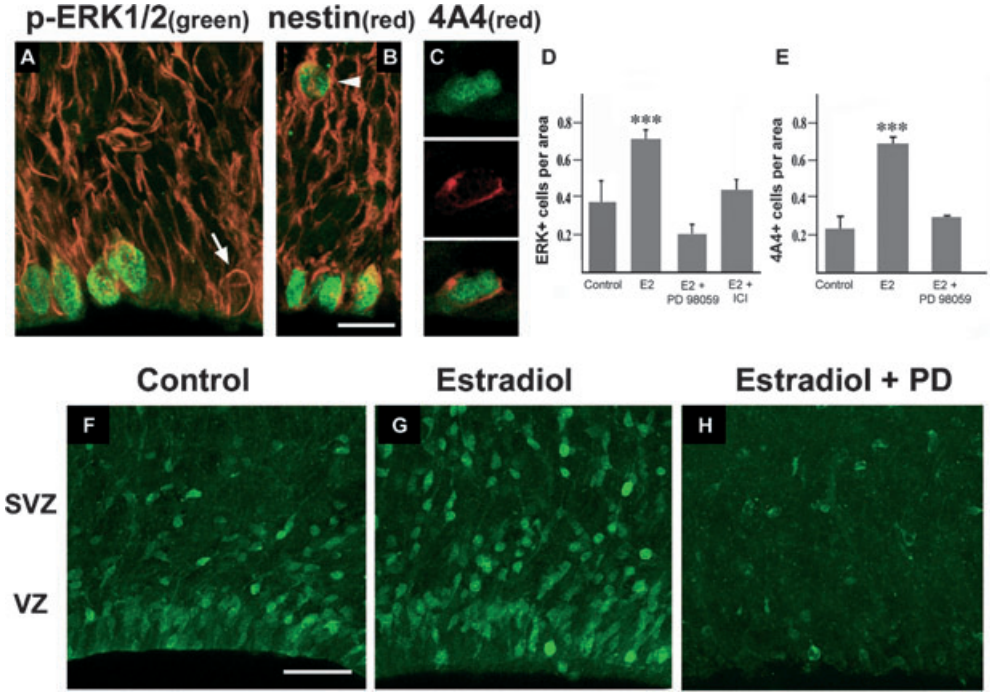

FIG. 9. E2 promoted activation of the phosphorylated MAPK ERK1/2. (A–C) p-ERK$^+$ cells *in vivo* (green) are located at the ventricular surface and also away from the ventricular surface (arrowhead). p-ERK$^+$ cells express nestin (red, A and B), which encapsulates the nuclei of these cells. Not all nestin$^+$ cells express p-ERK (arrow). (C) p-ERK$^+$ cells also express the mitotic radial glial marker 4A4 (red). (D and E) E2 treatment in culture significantly increased the number of p-ERK$^+$ cells. This increase was reversed by the ERK-specific blocker PD-98059 and the ER blocker ICI. E2 treatment also significantly increased the number of 4A4$^+$ cells and was reversed by ICI. (F–H) p-ERK$^+$ cells in the embryonic cortex of control, E2, and E2 plus the MAPK inhibitor PD 98059. Scale bars, 15 μm (in B for A–C), 50 μm (in F for F–H). ***$P < 0.001$.

intracellular signaling cascades of E2 in the developing brain (Ivanova *et al*., 2002).

Organotypic slice cultures were prepared from E15 neocortex and 100 nM E2 was included in the culture medium. BrdU (5 μM) was added to the medium for the last hour of the culture period. After 3 h, slices were fixed and immunostained with 4A4 or BrdU antibodies to test for the presence of mitotic neocortical progenitor cells. We found that 3 h of E2 treatment significantly increased the number of 4A4$^+$ mitotic cells in the VZ and SVZ of E15 neocortex (203%; $P < 0.001$, $n = 10$ experiments; Fig. 6, A1 and 2). The E2 treatment also increased the number of S-phase cells in the proliferative zones (126%; $P < 0.05$, $n = 3$ experiments; Fig. 6, B1 and 2). Addition of the E2 receptor blockers tamoxifen or ICI to the culture medium significantly reduced the number of 4A4$^+$ cells (Fig. 6, A2), indicating that E2-stimulated proliferation was mediated through ERs.

We next examined whether the proliferative effect would also be apparent after longer E2 treatment. We repeated the experiment and quantified the number of 4A4$^+$ and BrdU$^+$ cells in organotypic slices after 3 days in culture. The longer E2 treatment significantly increased the number of 4A4$^+$ progenitor cells (145%; $P < 0.01$, $n = 5$ experiments; Fig. 6, A3), and BrdU$^+$ progenitor cells (180%; $P < 0.05$, $n = 3$ experiments; Fig. 6, B3) in the proliferative zones of E15 slice cultures. Addition of the E2 receptor blockers tamoxifen or ICI to the culture medium blocked E2s the proliferative effect of E2 (Fig. 6, A3).

As we detected the presence of active aromatase enzyme in the embryonic cortex, we reasoned that administration of a substrate molecule for aromatase, androstenedione, should lead to local E2 synthesis and subsequent proliferation. We found that application of 0.5 nM androstenedione to cultured slices for 3 h produced a significant increase in the number of 4A4$^+$ cells in the proliferative zones of embryonic cortex (294%; $P < 0.0001$, $n = 3$ experiments; Fig. 7A).

We next tested whether the *in vitro* results reflect active signaling mechanisms *in vivo*. We administered the ER blockers tamoxifen or ICI to embryos *in utero* during the peak phase of cortical neurogenesis. Tamoxifen, which is able to cross the placental and blood–brain barriers, was injected intraperitoneally (40 nM) to pregnant mothers at 15 days of gestation and embryos were perfused 5 h later. ICI does not cross the placenta, and was therefore injected (100 nM) into the lateral ventricles of E15 fetuses *in utero*. Embryos were perfused 5 h after the injection. Cortical tissue was immunostained with the 4A4 antibody and the numbers of 4A4$^+$ cells in the proliferative zones of tamoxifen-treated, ICI-treated and control embryos were quantified. We found that the 5-h tamoxifen treatment significantly decreased the number of 4A4$^+$ dividing cells in the VZ and SVZ compared to controls (VZ, decreased to 80.5%, $P < 0.01$; SVZ, 52.0%, $P < 0.01$; $n = 3$ experiments; Fig. 7, B1 and 2), and that ICI also significantly decreased the number of 4A4$^+$ cells (VZ, 63.0%, $P < 0.01$; SVZ, 48.0%, $P < 0.01$; $n = 3$ experiments; Fig. 7, B1 and 2).

We examined whether the increased proliferation that we observed after E2 treatment was produced by an increase in survival of the progenitor cells. Slices were cultured with E2 as described above and the number of apoptotic cells was quantified at the end of the culture period using a TUNEL assay. We found no significant change in the number of apoptotic cells after 3, 12 or 24 h or 3 days of E2 treatment ($n = 4$), indicating that the increase in mitosis was not influenced by a change in the rate of cell death (supplementary Fig. S4).

Finally, we tested whether the mitogenic effect of E2 on progenitor cells differed between male and female cerebral cortices. Double-blind experiments were performed as described above, and fetus gender was determined through PCR amplification to detect the Sry gene present

on the Y chromosome. We found no significant difference between the numbers of $4A4^+$ progenitor cells after E2 treatment in organotypic cortical slice cultures obtained from male and female fetuses ($n = 3$ experiments; supplementary Fig. S5).

E2 promoted entry into the cell cycle

To more closely examine the cell cycle effects of E2 on VZ and SVZ progenitor cells, we treated organotypic slices with E2 and tracked progenitor cells as they progressed through the cell cycle using S-phase and M-phase markers. Slices were cultured in medium containing E2 for 1, 2 or 3 h, and BrdU was added to the medium for the last hour of treatment. Slices were fixed and the numbers of S-phase ($BrdU^+$) and M-phase ($4A4^+$) cells were quantified. We found that 1 h of E2 treatment produced a large increase in the number of cells in S-phase (246%; $P < 1 \times 10^{-4}$, $n = 3$ experiments; Fig. 8A) while the number of M-phase cells did not change. After 2 h of E2 treatment the number of S-phase cells was still significantly greater than controls (151%, $P < 0.05$), but we noted a significant increase in the number of $4A4^+$ M-phase cells (212%, $P < 0.01$). After 3 h of E2 treatment, the number of S-phase cells was no longer significantly different from the control group but the number of M-phase cells remained significantly greater than controls (200%, $P < 0.01$). In addition, we described above that 3 days of E2 treatment significantly increased the number of $4A4^+$ and $BrdU^+$ progenitor cells in the proliferative zones of E15 slice cultures (Fig. 6A). Together, these data support the hypothesis that E2 treatment promotes an initial recruitment of embryonic cortical progenitor cells into the S-phase from either G1 or perhaps G0 (Fig. 8B), consistent with proposed models of E2-stimulated proliferation in other systems (Kimura *et al*., 1976; Brunner *et al*., 1989; Minucci *et al*., 1997).

E2 promoted proliferation through activation of MAPK ERK1/2

We first examined the normal distribution of the activated form of MAPK ERK1/2, p-ERK, cells in the embryonic cortex. $p\text{-}ERK^+$ cells were located at the edge of the ventricle where radial glial cells divide, and also in the SVZ. To determine whether the $p\text{-}ERK^+$ cells were progenitor cells we costained fixed tissue with p-ERK and nestin or 4A4 antibodies, and found that $p\text{-}ERK^+$ cells in the VZ and SVZ expressed both progenitor cell markers (Fig. 9A–C).

We tested whether the E2-stimulated increase in proliferation was mediated through MAPK activation by treating embryonic slice cultures with E2 for 1 h and quantifying the number of $p\text{-}ERK^+$ cells in the proliferative zones. E2 treatment significantly increased the number of $p\text{-}ERK^+$ cells (186%; $P < 0.001$, $n = 24$ embryos). This effect was abolished by addition of the ERK1/2-specific blocker PD-98059 or the ER blocker ICI 182,780 to the culture medium (Fig. 9D and F–H). In addition, we found that the ERK1/2-specific blocker PD-98059 drastically reduced the number of $4A4^+$ mitoses after E2 treatment ($P < 1 \times 10^{-7}$, $n = 24$ embryos; Fig. 9E). The largest increase in p-ERK immunostaining occurred in the SVZ where $4A4^+$ IP cells divide (Fig. 9G). These results demonstrate that E2 administration activates the MAPK ERK1/2, and that this intracellular pathway mediates the increase in cell proliferation seen after short E2 treatments.

Discussion

E2 increases proliferation in the embryonic neocortex

We found that E2 treatment rapidly increased proliferation *in vitro* during embryonic cortical neurogenesis. This effect was mediated by ERs as it was inhibited by the ER blockers tamoxifen and ICI. We also found that administration of tamoxifen and ICI to the embryonic cerebral cortex *in utero* significantly decreased proliferation. In addition, androstenedione also increased proliferation *in vitro*. Our findings are consistent with previous demonstrations that E2 stimulates proliferation of neural stem cells (Brannvall *et al*., 2002) and generation of new neurons in the adult hippocampus (Brannvall *et al*., 2002). We demonstrated that E2 increased proliferation in the embryonic neocortex after 3 h or 3 days of treatment. Tanapat *et al*. (2005) reported that a single administration of E2 to adult female ovariectomized rats increased neurogenesis in the dentate gyrus, but that neither pulsatile E2 administration to replicate the changing serum E2 level in cycling females nor long-term exposure for 2 or 3 weeks in ovariectomized females produced a significant increase in neurogenesis. However, progenitor cells in the embryonic cortex encounter an environment that differs from that in adult cortex in several important respects. First, maternal serum E2 concentration increases 50-fold over prepregnancy values (Taylor *et al*., 2001), reaching concentrations as high as 25 000 pg/mL in the human (Troisi *et al*., 2003). Second, serum E2 levels in the developing embryo do not fluctuate as they do in the estrous female. Third, and perhaps most important, the synthesis of E2 in proliferative regions of the embryonic cortex provides a local supply of E2 during embryonic cortical development. In this way, E2 can exert a steady influence on embryonic cortical progenitor cells and this could contribute to the high levels of proliferation in the developing cortex.

Our results suggest that E2 recruits cells into the S-phase from either G1 or G0, which would account for the large increase of S-phase cells that we observed after only 1 h of E2 treatment. Furthermore, E2 exerts a long-term effect on proliferation by reducing the length of the G1 phase (Kimura *et al*., 1976; Schatz *et al*., 1984; Brunner *et al*., 1989; Minucci *et al*., 1997). For example, the ER blocker tamoxifen reduces proliferation of human breast cancer cells by greatly increasing the G1 phase (Brunner *et al*., 1989). Taken together, these data support the idea that E2 increases the rate of proliferation in dividing cortical progenitor cells by shortening the G1 phase.

Work in the adult hippocampus has shown that ERα stimulation can work both in a paracrine and an autocrine fashion (Prange-Kiel *et al*., 2003). In the developing zebrafish, E2 acts through ERs at very early stages of development to activate the aromatase gene, resulting in the local production of E2 by radial glial cells (Menuet *et al*., 2005). Thus E2 acts in a positive feedback loop to stimulate production of local E2 in the teleost VZ. We show that mammalian VZ cells express both ERα and aromatase; it is thus possible that E2 exerts both paracrine and autocrine effects in the embryonic VZ.

Our findings support the hypothesis that E2 serves as a proliferative agent in the developing cortex. Defects in proliferation are known to produce cortical abnormalities such as lissencephaly, but these have not been reported for aromatase knockout (ArKO) or ERαKO mice. However, heterozygote pairs are used to produce the ArKO mice as adult ArKO mice do not successfully mate. As a result the developing brain of ArKO embryos is exposed to maternal E2 during gestation. Furthermore, the ArKO forebrain exhibits a greater than two-fold increase in ERα expression (Agarwal *et al*., 2000), which may render brain cells more sensitive to maternal E2. ERαKO mice also exhibit significant changes that include increased expression of ERβ and an increased activation of intracellular signaling components following E2 stimulation (Toran-Allerand *et al*., 1999), which could compensate for the loss of ERα receptors. Therefore, the ArKO and ERαKO mice may not fully, or cleanly, address the role of E2 signaling during embryonic neocortical development.

Active aromatase is present in the proliferative populations of the developing cerebral cortex

We have shown that key requisite components of the E2 signaling system are present in the embryonic cortex. Active aromatase is expressed by radial glial cells and IP cells during cortical neurogenesis. Our findings are in agreement with previous reports demonstrating the presence of aromatase mRNA in the embryonic neocortex (Harada & Yamada, 1992), and by recent demonstrations that radial glial cells express aromatase protein and mRNA in the adult teleost and zebrafinch brain (Forlano *et al*., 2001; Menuet *et al*., 2003, 2005; Peterson *et al*., 2004). Radial glial cells serve as neural progenitor cells in several species including rodents and birds (Alvarez-Buylla *et al*., 1990; Malatesta *et al*., 2000; Miyata *et al*., 2001; Noctor *et al*., 2001). Interestingly, aromatase expression has been demonstrated in other neurogenic regions of the brain such as the adult hippocampus (Hojo *et al*., 2004), and has been correlated with seasonal neurogenesis in the adult songbird (Schlinger & Arnold, 1991; Schlinger, 1997). Together these findings are consistent with the hypothesis that E2 is synthesized locally in neurogenic regions of the developing and adult brain.

ERα is present in the proliferative populations of the embryonic neocortex

We have demonstrated that radial glial cells in the neocortical VZ express ERα in the cytoplasm and membrane during embryonic neurogenesis. Our results are supported by previous data showing that ERs are expressed in the embryonic cortex (Vito & Fox, 1979; Friedman *et al*., 1983), in proliferating cells in the dentate gyrus (Perez-Martin *et al*., 2003) and in embryonic and adult stem cells (Brannvall *et al*., 2002). In addition, ERα expression decreases during the postnatal period while ERβ expression increases (Donahue *et al*., 2000; Blurton-Jones & Tuszynski, 2001; Milner *et al*., 2001; Zsarnovszky & Belcher, 2001; Mitra *et al*., 2003; Perez *et al*., 2003). The change in the relative abundance of ERα and ERβ coincides with the end of cortical neurogenesis. This switch is consistent with the hypothesis that ERα expression is correlated with proliferation while ERβ expression is correlated with cellular differentiation (Omoto *et al*., 2005). Recent studies have shown that neural progenitor cells in the adult SVZ and dentate gyrus express ERα (Isgor & Watson, 2005), and that putative breast stem cells express high levels of ERα (Clarke *et al*., 2005). Together these data indicate that ERα expression is common to neural progenitor cells and stem cells in both embryo and adult, supporting the idea that estradiol is an important regulator of proliferation.

E2 promotes proliferation through MAPK ERK1/2

Cellular responses to E2 are related to the type and cellular location of ERs. Nuclear ERs initiate the classical genomic response by binding to estrogen response elements, while cytoplasmic or membrane-bound receptors can activate either the genomic or rapid nongenomic signaling pathways (Simoncini *et al*., 2004). E2 stimulation of the nongenomic pathway activates the MAPK ERK1/2 (Singer *et al*., 1999; Singh *et al*., 1999). p-ERK promotes several important cellular functions including proliferation (Razandi *et al*., 2003). We found that short E2 treatment prompted a significant increase in the levels of p-ERK in the proliferative zones and also increased the number of mitotic progenitor cells. The ERK1/2-specific blocker PD-98059 abolished the increase in p-ERK, and also abolished the proliferative effect of E2. These results, together with our finding that the majority of VZ cells express cytoplasmic and membrane ERα, indicate that E2 acts through cytoplasmic- and membrane-bound receptors and the MAPK pathway to promote progenitor cells to enter the S-phase. This interpretation is consistent with previous reports showing that membrane ERα receptors activate p-ERK, stimulate entry into the S-phase and initiate expression of the cell cycle regulator cyclin D1 (Razandi *et al*., 2003). Several pathways can lead to ERK1/2 activation through phosphorylation by ERK kinases (Setalo *et al*., 2002) such as PKC (Setalo *et al*., 2005), IP3 (Ivanova *et al*., 2002) and cAMP (Boulware *et al*., 2005), and increased levels of intracellular Ca^{2+} (Zhao *et al*., 2005). Future studies should address the intracellular estradiol signaling pathway(s) that activate ERK1/2 and promote proliferation in the developing cerebral cortex.

AFP differentially regulates E2 in the developing neocortex

We found strong AFP expression in the embryonic VZ but not in the SVZ. AFP is synthesized in several fetal organs, but the regional AFP expression we observe most probably reflects local synthesis in the cortex. AFP mRNA transcripts have been detected in the brain of rodents (McLeod & Cooke, 1989) but not baboons (Naval *et al*., 1992). However, variable forms of AFP mRNA transcripts have been detected in other primate brains including human (Kubota *et al*., 2002), suggesting that this protein may be expressed in the brain of many species. AFP binding of E2 has been well documented in rodent (Attardi & Ruoslahti, 1976) but there has been debate concerning whether human AFP binds E2. Early work indicated that human AFP lacked E2-binding activity (Nunez *et al*., 1974; Ali *et al*., 1981). However, more recent work has described in detail both the estradiol-binding regions of human AFP and the inhibitory actions of AFP-bound E2 (for review see Mizejewski *et al*., 2004). AFP regulates the amount of free unbound E2 (Attardi & Ruoslahti, 1976), and has been proposed as inhibiting the proliferation of E2-sensitive cells (Soto & Sonnenschein, 1980; Mizejewski *et al*., 2004).

We propose that AFP may differentially regulate the effects of E2 in the embryonic proliferative zones. High levels of AFP in the VZ would be expected to reduce the amount of free E2, thereby reducing E2-promoted stimulation. This in turn could contribute to the longer cell cycle times reported for VZ cells (Takahashi *et al*., 1995). In contrast, low levels of AFP in the embryonic SVZ may allow E2 to exert a stronger stimulatory effect on IP cells, producing the shorter cell cycle exhibited by these cells (Takahashi *et al*., 1995). Progenitor cells in the VZ and SVZ have been reported to undergo different modes of division; VZ cells are more likely to divide asymmetrically and SVZ cells are more likely to divide symmetrically (Haubensak *et al*., 2004; Noctor *et al*., 2004). Modulation of the progenitor cell cycle in the VZ and SVZ through E2 signaling may therefore influence the proportion of symmetric and asymmetric divisions in the cortical proliferative zones, contributing to the proper cellular composition of the cerebral cortex.

Methodological implications

Tamoxifen is a widely used tool in clinical and research applications. Many gene expression studies using the Cre/loxP system have incorporated Cre recombinase fused with a mutated ligand-binding domain of the human ERs that binds tamoxifen but not E2. Tamoxifen administration initiates gene expression in these studies. Blockade of ERs with tamoxifen increases the length of the G1 phase of the cell cycle, which reduces the proliferation of mitotic cells (Brunner *et al*., 1989). The presence of ERs in the normal embryonic neocortex indicates that tamoxifen treatment has the potential to alter the normal

rate of proliferation in addition to activating targeted gene expression. Future studies using tamoxifen-dependent Cre recombinase to regulate gene expression should consider the potential for undesired variables and closely monitor normally dividing cell populations.

Clinical implications

Man-made chemicals, known as xenoestrogens, such as DDT and PCB, and naturally occurring substances known as phytoestrogens, bind to ERs and have estradiol-like properties. Xenoestrogens are present in industrial pollutants, canned foods, dental sealants and many others (Zsarnovszky & Belcher, 2001). The actions of phytoestrogens are poorly understood. However, they have been promoted in the popular press as protective agents for some forms of cancer, cardiovascular disease, osteoporosis and menopausal symptoms (Zsarnovszky & Belcher, 2001). Consequently, even though it has not been scientifically demonstrated, phytoestrogens are promoted as safe and beneficial natural dietary supplements. Phytoestrogens are present at high levels in soy-based dietary supplements and also in infant formula. Phytoestrogens are widely used and are one of the most frequently recovered contaminants in amniotic fluid of pregnant women in the US (Foster *et al*., 2000), exposing developing fetuses to high levels of these exogenous E2-mimicking agents during brain development. The potential mechanism of action(s) and the effects of xenoestrogen and phytoestrogen exposure are poorly understood. Knowing the cellular mechanisms by which endogenous E2 promotes proliferation of neurogenic progenitor cells in the developing brain is crucially important. Future studies should address the effects of endogenous and exogenous estrogens on proliferation in the developing cerebral cortex.

Supplementary material

The following supplementary material may be found on www.blackwell-synergy.com

Fig. S1. Radial glial cells in the cortical ventricular zone expressed aromatase.

Fig. S2. ERα was expressed in the VZ and SVZ of the developing cerebral cortex.

Fig. S3. Nuclear and cytoplasmic ERα were expressed in embryonic VZ and cortical plate cells.

Fig. S4. E2 treatment did not increase the number of apoptotic cells in the VZ and SVZ of E15 developing mouse cortex.

Fig. S5. E2 treatment *in vitro* affected proliferation in male and female developing neocortex equally.

Acknowledgements

We thank Drs Iñigo Azcoitia, Synthia Mellon, Toran-Allerand and members of the Kriegstein lab for helpful comments on the manuscript; Drs Hutchison, Bicknell, Harada and Webb for providing reagents; and Joy Mirjahangir, Xiaoyan Wang, Jeanelle Agudelo, Frances Denoto-Reynolds, David Castañeda-Castellanos, Minoree Kohwi, Kevin Corbit, Elena Demireva, and William Walantus for technical assistance and advice. This work was supported by a grant from the Ministerio de Educación, Cultura y Deporte, Spain to V.M.C., and National Institutes of Health grants NS21223 and NS35710 to A.R.K.

Abbreviations

ACSF, artificial cerebrospinal fluid; AFP, α-fetoprotein; BrdU, bromodeoxyuridine; E, embryonic day; E2, 17-β-estradiol; eGFP, enhanced green fluorescent protein; ERα, estrogen receptor-α; ERβ, estrogen receptor-β; ERK1/2, extracellular signal-regulated kinase 1/2; IP, intermediate progenitor; MAPK, mitogen-activated protein kinase; p-, phospho-; P, postnatal day; p-ERK, phosphorylated ERK1/2; PFA, 4% paraformaldehyde in phosphate-buffered saline; SVZ, subventricular zone; VZ, ventricular zone.

References

Adinolfi, M., Beck, S.E., Haddad, S.A. & Seller, M.J. (1976) Permeability of the blood–cerebrospinal fluid barrier to plasma proteins during foetal and perinatal life. *Nature*, **259**, 140–141.

Adinolfi, M. & Haddad, S.A. (1977) Levels of plasma proteins in human and rat fetal CSF and the development of the blood–CSF barrier. *Neuropadiatrie*, **8**, 345–353.

Agarwal, V.R., Sinton, C.M., Liang, C., Fisher, C., German, D.C. & Simpson, E.R. (2000) Upregulation of estrogen receptors in the forebrain of aromatase knockout (ArKO) mice. *Mol. Cell Endocrinol.*, **162**, 9–16.

Ali, M., Balapure, A.K., Singh, D.R., Shukla, R.N. & Sahib, M.K. (1981) Ontogeny of alpha-foetoprotein in human foetal brain. *Brain Res.*, **207**, 459–464.

Alvarez-Buylla, A., Theelen, M. & Nottebohm, F. (1990) Proliferation 'hot spots' in adult avian ventricular zone reveal radial cell division. *Neuron*, **5**, 101–109.

Attardi, B. & Ruoslahti, E. (1976) Foetoneonatal oestradiol-binding protein in mouse brain cytosol is alpha foetoprotein. *Nature*, **263**, 685–687.

Balthazart, J., Foidart, A. & Harada, N. (1990) Immunocytochemical localization of aromatase in the brain. *Brain Res.*, **514**, 327–333.

Balthazart, J., Foidart, A., Surlemont, C. & Harada, N. (1991) Distribution of aromatase-immunoreactive cells in the mouse forebrain. *Cell Tissue Res.*, **263**, 71–79.

Balthazart, J., Tlemcani, O. & Harada, N. (1996) Localization of testosterone-sensitive and sexually dimorphic aromatase-immunoreactive cells in the quail preoptic area. *J. Chem. Neuroanat.*, **11**, 147–171.

Banasr, M., Hery, M., Brezun, J.M. & Daszuta, A. (2001) Serotonin mediates oestrogen stimulation of cell proliferation in the adult dentate gyrus. *Eur. J. Neurosci.*, **14**, 1417–1424.

Beyer, C. (1999) Estrogen and the developing mammalian brain. *Anat. Embryol. (Berl.)*, **199**, 379–390.

Beyer, C., Tramonte, R., Hutchison, R.E., Sharp, P.J., Barker, P.J., Huskisson, N.S. & Hutchison, J.B. (1994) Aromatase-immunoreactive neurons in the adult female chicken brain detected using a specific antibody. *Brain Res. Bull.*, **33**, 583–588.

Blaustein, J.D. (1992) Cytoplasmic estrogen receptors in rat brain: immunocytochemical evidence using three antibodies with distinct epitopes. *Endocrinology*, **131**, 1336–1342.

Blaustein, J.D. & Turcotte, J.C. (1989) Estrogen receptor-immunostaining of neuronal cytoplasmic processes as well as cell nuclei in guinea pig brain. *Brain Res.*, **495**, 75–82.

Blurton-Jones, M. & Tuszynski, M.H. (2001) Reactive astrocytes express estrogen receptors in the injured primate brain. *J. Comp. Neurol.*, **433**, 115–123.

Boulware, M.I., Weick, J.P., Becklund, B.R., Kuo, S.P., Groth, R.D. & Mermelstein, P.G. (2005) Estradiol activates group I and II metabotropic glutamate receptor signaling, leading to opposing influences on cAMP response element-binding protein. *J. Neurosci.*, **25**, 5066–5078.

Brannvall, K., Korhonen, L. & Lindholm, D. (2002) Estrogen-receptor-dependent regulation of neural stem cell proliferation and differentiation. *Mol. Cell Neurosci.*, **21**, 512–520.

Brunner, N., Bronzert, D., Vindelov, L.L., Rygaard, K., Spang-Thomsen, M. & Lippman, M.E. (1989) Effect on growth and cell cycle kinetics of estradiol and tamoxifen on MCF-7 human breast cancer cells grown in vitro and in nude mice. *Cancer Res.*, **49**, 1515–1520.

Clarke, R.B. (2004) Human breast cell proliferation and its relationship to steroid receptor expression. *Climacteric*, **7**, 129–137.

Clarke, R.B., Spence, K., Anderson, E., Howell, A., Okano, H. & Potten, C.S. (2005) A putative human breast stem cell population is enriched for steroid receptor-positive cells. *Dev. Biol.*, **277**, 443–456.

Connolly, P.B., Roselli, C.E. & Resko, J.A. (1994) Aromatase activity in developing guinea pig brain: ontogeny and effects of exogenous androgens. *Biol. Reprod.*, **50**, 436–441.

Dellovade, T.L., Rissman, E.F., Thompson, N., Harada, N. & Ottinger, M.A. (1995) Co-localization of aromatase enzyme and estrogen receptor immunoreactivity in the preoptic area during reproductive aging. *Brain Res.*, **674**, 181–187.

Donahue, J.E., Stopa, E.G., Chorsky, R.L., King, J.C., Schipper, H.M., Tobet, S.A., Blaustein, J.D. & Reichlin, S. (2000) Cells containing immunoreactive estrogen receptor-alpha in the human basal forebrain. *Brain Res.*, **856**, 142–151.

Englund, C., Fink, A., Lau, C., Pham, D., Daza, R.A., Bulfone, A., Kowalczyk, T. & Hevner, R.F. (2005) Pax6, Tbr2, and Tbr1 are expressed sequentially by radial glia, intermediate progenitor cells, and postmitotic neurons in developing neocortex. *J. Neurosci.*, **25**, 247–251.

Fatehi, M., Kombian, S.B. & Saleh, T.M. (2005) 17Beta-estradiol inhibits outward potassium currents recorded in rat parabrachial nucleus cells in vitro. *Neuroscience*, **135**, 1075–1086.

Foidart, A., Harada, N. & Balthazart, J. (1995) Aromatase-immunoreactive cells are present in mouse brain areas that are known to express high levels of aromatase activity. *Cell Tissue Res.*, **280**, 561–574.

Forlano, P.M., Deitcher, D.L., Myers, D.A. & Bass, A.H. (2001) Anatomical distribution and cellular basis for high levels of aromatase activity in the brain of teleost fish: aromatase enzyme and mRNA expression identify glia as source. *J. Neurosci.*, **21**, 8943–8955.

Foster, W., Chan, S., Platt, L. & Hughes, C. (2000) Detection of endocrine disrupting chemicals in samples of second trimester human amniotic fluid. *J. Clin. Endocrinol. Metab.*, **85**, 2954–2957.

Friedman, W.J., McEwen, B.S., Toran-Allerand, C.D. & Gerlach, J.L. (1983) Perinatal development of hypothalamic and cortical estrogen receptors in mouse brain: methodological aspects. *Brain Res.*, **313**, 19–27.

Garcia-Segura, L.M., Wozniak, A., Azcoitia, I., Rodriguez, J.R., Hutchison, R.E. & Hutchison, J.B. (1999) Aromatase expression by astrocytes after brain injury: implications for local estrogen formation in brain repair. *Neuroscience*, **89**, 567–578.

Harada, N. (1988) Novel properties of human placental aromatase as cytochrome P-450: purification and characterization of a unique form of aromatase. *J. Biochem. (Tokyo)*, **103**, 106–113.

Harada, N. & Yamada, K. (1992) Ontogeny of aromatase messenger ribonucleic acid in mouse brain: fluorometrical quantitation by polymerase chain reaction. *Endocrinology*, **131**, 2306–2312.

Haubensak, W., Attardo, A., Denk, W. & Huttner, W.B. (2004) Neurons arise in the basal neuroepithelium of the early mammalian telencephalon: a major site of neurogenesis. *Proc. Natl Acad. Sci. USA*, **101**, 3196–3201.

Hojo, Y., Hattori, T.A., Enami, T., Furukawa, A., Suzuki, K., Ishii, H.T., Mukai, H., Morrison, J.H., Janssen, W.G., Kominami, S., Harada, N., Kimoto, T. & Kawato, S. (2004) Adult male rat hippocampus synthesizes estradiol from pregnenolone by cytochromes P45017alpha and P450 aromatase localized in neurons. *Proc. Natl Acad. Sci. USA*, **101**, 865–870.

Hutchison, J.B., Beyer, C., Hutchison, R.E. & Wozniak, A. (1997) Sex differences in the regulation of embryonic brain aromatase. *J. Steroid Biochem. Mol. Biol.*, **61**, 315–322.

Isgor, C. & Watson, S.J. (2005) Estrogen receptor alpha and beta mRNA expressions by proliferating and differentiating cells in the adult rat dentate gyrus and subventricular zone. *Neuroscience*, **134**, 847–856.

Ivanova, T., Mendez, P., Garcia-Segura, L.M. & Beyer, C. (2002) Rapid stimulation of the PI3-kinase/Akt signalling pathway in developing midbrain neurones by oestrogen. *J. Neuroendocrinol.*, **14**, 73–79.

Jakab, R.L., Horvath, T.L., Leranth, C., Harada, N. & Naftolin, F. (1993) Aromatase immunoreactivity in the rat brain: gonadectomy-sensitive hypothalamic neurons and an unresponsive 'limbic ring' of the lateral septum-bed nucleus-amygdala complex. *J. Steroid Biochem. Mol. Biol.*, **44**, 481–498.

Kamei, Y., Inagaki, N., Nishizawa, M., Tsutsumi, O., Taketani, Y. & Inagaki, M. (1998) Visualization of mitotic radial glial lineage cells in the developing rat brain by Cdc2 kinase-phosphorylated vimentin. *Glia*, **23**, 191–199.

Kimura, J., Obata, T. & Okada, H. (1976) Kinetic analysis of hormone-induced mitoses in epithelial cells of mouse uterus and vagina. *Endocrinol. Jpn*, **23**, 391–399.

Kubota, H., Storms, R.W. & Reid, L.M. (2002) Variant forms of alpha-fetoprotein transcripts expressed in human hematopoietic progenitors. Implications for their developmental potential towards endoderm. *J. Biol. Chem.*, **277**, 27629–27635.

Lehman, M.N., Ebling, F.J., Moenter, S.M. & Karsch, F.J. (1993) Distribution of estrogen receptor-immunoreactive cells in the sheep brain. *Endocrinology*, **133**, 876–886.

Letinic, K., Zoncu, R. & Rakic, P. (2002) Origin of GABAergic neurons in the human neocortex. *Nature*, **417**, 645–649.

MacLusky, N.J., Naftolin, F. & Goldman-Rakic, P.S. (1986) Estrogen formation and binding in the cerebral cortex of the developing rhesus monkey. *Proc. Natl Acad. Sci. USA*, **83**, 513–516.

Malatesta, P., Hartfuss, E. & Gotz, M. (2000) Isolation of radial glial cells by fluorescent-activated cell sorting reveals a neuronal lineage. *Development*, **127**, 5253–5263.

McCall, A.L., Han, S.J., Millington, W.R. & Baum, M.J. (1981) Non-saturable transport of [^{3}H]oestradiol across the blood–brain barrier in female rats is reduced by neonatal serum. *J. Reprod. Fertil.*, **61**, 103–108.

McEwen, B.S., Coirini, H., Westlind-Danielsson, A., Frankfurt, M., Gould, E., Schumacher, M. & Woolley, C. (1991) Steroid hormones as mediators of neural plasticity. [Review]. *J. Steroid Biochem. Mol. Biol.*, **39**, 223–232.

McLeod, J.F. & Cooke, N.E. (1989) The vitamin D-binding protein, alpha-fetoprotein, albumin multigene family: detection of transcripts in multiple tissues. *J. Biol. Chem.*, **264**, 21760–21769.

Menuet, A., Anglade, I., Le Guevel, R., Pellegrini, E., Pakdel, F. & Kah, O. (2003) Distribution of aromatase mRNA and protein in the brain and pituitary of female rainbow trout: Comparison with estrogen receptor alpha. *J. Comp. Neurol.*, **462**, 180–193.

Menuet, A., Pellegrini, E., Brion, F., Gueguen, M.M., Anglade, I., Pakdel, F. & Kah, O. (2005) Expression and estrogen-dependent regulation of the zebrafish brain aromatase gene. *J. Comp. Neurol.*, **485**, 304–320.

Milner, T.A., McEwen, B.S., Hayashi, S., Li, C.J., Reagan, L.P. & Alves, S.E. (2001) Ultrastructural evidence that hippocampal alpha estrogen receptors are located at extranuclear sites. *J. Comp. Neurol.*, **429**, 355–371.

Minucci, S., Di Matteo, L., Chieffi, P., Pierantoni, R. & Fasano, S. (1997) 17 Beta-estradiol effects on mast cell number and spermatogonial mitotic index in the testis of the frog, *Rana esculenta*. *J. Exp. Zool.*, **278**, 93–100.

Mitra, S.W., Hoskin, E., Yudkovitz, J., Pear, L., Wilkinson, H.A., Hayashi, S., Pfaff, D.W., Ogawa, S., Rohrer, S.P., Schaeffer, J.M., McEwen, B.S. & Alves, S.E. (2003) Immunolocalization of estrogen receptor beta in the mouse brain: comparison with estrogen receptor alpha. *Endocrinology*, **144**, 2055–2067.

Miyata, T., Kawaguchi, A., Okano, H. & Ogawa, M. (2001) Asymmetric inheritance of radial glial fibers by cortical neurons. *Neuron*, **31**, 727–741.

Miyata, T., Kawaguchi, A., Saito, K., Kawano, M., Muto, T. & Ogawa, M. (2004) Asymmetric production of surface-dividing and non-surface-dividing cortical progenitor cells. *Development*, **131**, 3133–3145.

Mizejewski, G., Smith, G. & Butterstein, G. (2004) Review and proposed action of alpha-fetoprotein growth inhibitory peptides as estrogen and cytoskeleton-associated factors. *Cell Biol. Int.*, **28**, 913–933.

Naval, J., Calvo, M., Laborda, J., Dubouch, P., Frain, M., Sala-Trepat, J.M. & Uriel, J. (1992) Expression of mRNAs for alpha-fetoprotein (AFP) and albumin and incorporation of AFP and docosahexaenoic acid in baboon fetuses. *J. Biochem. (Tokyo)*, **111**, 649–654.

Noctor, S.C., Flint, A.C., Weissman, T.A., Dammerman, R.S. & Kriegstein, A.R. (2001) Neurons derived from radial glial cells establish radial units in neocortex. *Nature*, **409**, 714–720.

Noctor, S.C., Martinez-Cerdeno, V., Ivic, L. & Kriegstein, A.R. (2004) Cortical neurons arise in symmetric and asymmetric division zones and migrate through specific phases. *Nat. Neurosci.*, **7**, 136–144.

Nunez, E., Vallette, G., Benassayag, C. & Jayle, M.F. (1974) Comparative study on the binding of estrogens by human and rat serum proteins in development. *Biochem. Biophys. Res. Commun*, **57**, 126–133.

Omoto, Y., Imamov, O., Warner, M. & Gustafsson, J.A. (2005) Estrogen receptor {alpha} and imprinting of the neonatal mouse ventral prostate by estrogen. *Proc. Natl Acad. Sci. USA*, **102**, 1484–1489.

Ormerod, B.K., Lee, T.T. & Galea, L.A. (2004) Estradiol enhances neurogenesis in the dentate gyri of adult male meadow voles by increasing the survival of young granule neurons. *Neuroscience*, **128**, 645–654.

Osman, N.M., Naora, H. & Otani, H. (2004) Glycosyltransferase encoding gene EXTL3 is differentially expressed in the developing and adult mouse cerebral cortex. *Brain Res. Dev. Brain Res.*, **151**, 111–117.

Parikh, I., Rajendran, K.G., Su, J.L., Lopez, T. & Sar, M. (1987) Are estrogen receptors cytoplasmic or nuclear? Some immunocytochemical and biochemical studies. *J. Steroid Biochem.*, **27**, 185–192.

Perez, S.E., Chen, E.Y. & Mufson, E.J. (2003) Distribution of estrogen receptor alpha and beta immunoreactive profiles in the postnatal rat brain. *Brain Res. Dev. Brain Res.*, **145**, 117–139.

Perez-Martin, M., Azcoitia, I., Trejo, J.L., Sierra, A. & Garcia-Segura, L.M. (2003) An antagonist of estrogen receptors blocks the induction of adult neurogenesis by insulin-like growth factor-I in the dentate gyrus of adult female rat. *Eur. J. Neurosci.*, **18**, 923–930.

Peterson, R.S., Lee, D.W., Fernando, G. & Schlinger, B.A. (2004) Radial glia express aromatase in the injured zebra finch brain. *J. Comp. Neurol.*, **475**, 261–269.

Pietras, R.J. & Szego, C.M. (1977) Specific binding sites for oestrogen at the outer surfaces of isolated endometrial cells. *Nature*, **265**, 69–72.

Prange-Kiel, J., Wehrenberg, U., Jarry, H. & Rune, G.M. (2003) Para/autocrine regulation of estrogen receptors in hippocampal neurons. *Hippocampus*, **13**, 226–234.

Razandi, M., Alton, G., Pedram, A., Ghonshani, S., Webb, P. & Levin, E.R. (2003) Identification of a structural determinant necessary for the localization

and function of estrogen receptor alpha at the plasma membrane. *Mol. Cell Biol.*, **23**, 1633–1646.
Reynolds, M.L., Mollgard, K. & Saunders, N.R. (1983) The distribution of plasma proteins during early embryonic development in the sheep. *Anat. Embryol (Berl.)*, **168**, 227–240.
Sasano, H., Takashashi, K., Satoh, F., Nagura, H. & Harada, N. (1998) Aromatase in the human central nervous system. *Clin. Endocrinol. (Oxf.)*, **48**, 325–329.
Schatz, R., Soto, A.M. & Sonnenschein, C. (1984) Estrogen-induced cell multiplication: direct or indirect effect on rat uterine cells? *Endocrinology*, **115**, 501–506.
Schlinger, B.A. (1997) Sex steroids and their actions on the birdsong system. *J. Neurobiol.*, **33**, 619–631.
Schlinger, B.A. & Arnold, A.P. (1991) Brain is the major site of estrogen synthesis in a male songbird. *Proc. Natl Acad. Sci. USA*, **88**, 4191–4194.
Schmechel, D.E. & Rakic, P. (1979) Arrested proliferation of radial glial cells during midgestation in rhesus monkey. *Nature*, **277**, 303–305.
Setalo, G. Jr, Singh, M., Guan, X. & Toran-Allerand, C.D. (2002) Estradiol-induced phosphorylation of ERK1/2 in explants of the mouse cerebral cortex: the roles of heat shock protein 90 (Hsp90) and MEK2. *J. Neurobiol.*, **50**, 1–12.
Setalo, G. Jr, Singh, M., Nethrapalli, I.S. & Toran-Allerand, C.D. (2005) Protein kinase C activity is necessary for estrogen-induced Erk phosphorylation in neocortical explants. *Neurochem. Res.*, **30**, 779–790.
Simoncini, T., Mannella, P., Fornari, L., Caruso, A., Varone, G. & Genazzani, A.R. (2004) Genomic and non-genomic effects of estrogens on endothelial cells. *Steroids*, **69**, 537–542.
Singer, C.A., Figueroa-Masot, X.A., Batchelor, R.H. & Dorsa, D.M. (1999) The mitogen-activated protein kinase pathway mediates estrogen neuroprotection after glutamate toxicity in primary cortical neurons. *J. Neurosci.*, **19**, 2455–2463.
Singh, M., Setalo, G. Jr, Guan, X., Warren, M. & Toran-Allerand, C.D. (1999) Estrogen-induced activation of mitogen-activated protein kinase in cerebral cortical explants: convergence of estrogen and neurotrophin signaling pathways. *J. Neurosci.*, **19**, 1179–1188.
Soto, A.M. & Sonnenschein, C. (1980) Control of growth of estrogen-sensitive cells: role for alpha-fetoprotein. *Proc. Natl Acad. Sci. USA*, **77**, 2084–2087.
Takahashi, T., Nowakowski, R.S. & Caviness, V. Jr (1995) The cell cycle of the pseudostratified ventricular epithelium of the embryonic murine cerebral wall. *J. Neurosci.*, **15**, 6046–6057.
Tamamaki, N., Nakamura, K., Okamoto, K. & Kaneko, T. (2001) Radial glia is a progenitor of neocortical neurons in the developing cerebral cortex. *Neurosci. Res.*, **41**, 51–60.
Tanapat, P., Hastings, N.B. & Gould, E. (2005) Ovarian steroids influence cell proliferation in the dentate gyrus of the adult female rat in a dose- and time-dependent manner. *J. Comp. Neurol.*, **481**, 252–265.
Tanapat, P., Hastings, N.B., Reeves, A.J. & Gould, E. (1999) Estrogen stimulates a transient increase in the number of new neurons in the dentate gyrus of the adult female rat. *J. Neurosci.*, **19**, 5792–5801.
Tarabykin, V., Stoykova, A., Usman, N. & Gruss, P. (2001) Cortical upper layer neurons derive from the subventricular zone as indicated by Svet1 gene expression. *Development*, **128**, 1983–1993.
Taylor, R.N., Lebovic, D.I. & Martin-Cadieux, M.C. (2001) The endocrinology of pregnancy. In: Greenspan, F.S. & Gardner, D.G., eds. *Basic and Clinical Endocrinology.* McGraw-Hill, New York, pp. 575–602.
Thompson, E.A. Jr & Siiteri, P.K. (1974) Utilization of oxygen and reduced nicotinamide adenine dinucleotide phosphate by human placental microsomes during aromatization of androstenedione. *J. Biol. Chem.*, **249**, 5364–5372.
Toran-Allerand, C.D. (2004) Estrogen and the brain: beyond ER-alpha and ER-beta. *Exp. Gerontol.*, **39**, 1579–1586.
Toran-Allerand, C.D., Guan, X., MacLusky, N.J., Horvath, T.L., Diano, S., Singh, M., Connolly, E.S. Jr, Nethrapalli, I.S. & Tinnikov, A.A. (2002) ER-X: a novel, plasma membrane-associated, putative estrogen receptor that is regulated during development and after ischemic brain injury. *J. Neurosci.*, **22**, 8391–8401.
Toran-Allerand, C.D., Singh, M. & Setalo, G. Jr (1999) Novel mechanisms of estrogen action in the brain: new players in an old story. *Front. Neuroendocrinol.*, **20**, 97–121.
Troisi, R., Potischman, N., Roberts, J.M., Harger, G., Markovic, N., Cole, B., Lykins, D., Siiteri, P. & Hoover, R.N. (2003) Correlation of serum hormone concentrations in maternal and umbilical cord samples. *Cancer Epidemiol. Biomarkers Prev.*, **12**, 452–456.
Vasudevan, N., Kow, L.M. & Pfaff, D. (2005) Integration of steroid hormone initiated membrane action to genomic function in the brain. *Steroids*, **70**, 388–396.
Vito, C.C. & Fox, T.O. (1979) Embryonic rodent brain contains estrogen receptors. *Science*, **204**, 517–519.
Zhao, L., Chen, S., Ming Wang, J. & Brinton, R.D. (2005) 17beta-estradiol induces Ca^{2+} influx, dendritic and nuclear Ca^{2+} rise and subsequent cyclic AMP response element-binding protein activation in hippocampal neurons: a potential initiation mechanism for estrogen neurotrophism. *Neuroscience*, **132**, 299–311.
Zsarnovszky, A. & Belcher, S.M. (2001) Identification of a developmental gradient of estrogen receptor expression and cellular localization in the developing and adult female rat primary somatosensory cortex. *Brain Res. Dev. Brain Res.*, **129**, 39–46.