Brain-synthesized oestrogens regulate cortical migration in a sexually divergent manner

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Abstract
Oestrogens play an important role in brain development where they have been implicated in controlling various cellular processes. Several lines of evidence have been presented showing that oestrogens can be synthesized locally within the brain. Studies have demonstrated that aromatase, the enzyme responsible for the conversion of androgens to oestrogens, is expressed during early development in both male and female cortices. Furthermore, 17β-oestradiol has been measured in foetal brain tissue from multiple species. 17β-oestradiol regulates neural progenitor proliferation as well as the development of early neuronal morphology. However, what role locally derived oestrogens play in regulating cortical migration and, moreover, whether these effects are the same in males and females are unknown. Here, we investigated the impact of knockdown expression of Cyp19a1, which encodes aromatase, between embryonic day (E) 14.5 and postnatal day 0 (P0) had on neural migration within the cortex. Aromatase was expressed in the developing cortex of both sexes, but at significantly higher levels in male than female mice. Under basal conditions, no obvious differences in cortical migration between male and female mice were observed. However, knockdown of Cyp19a1 resulted in an increase in cells within the cortical plate, and a concurrent decrease in the subventricular zone/ventricular zone in P0 male mice. Interestingly, the opposite effect was observed in females, who displayed a significant reduction in cells migrating to the cortical plate. Together, these findings indicate that brain-derived oestrogens regulate radial migration through distinct mechanisms in males and females.

KEYWORDS
aromatase, corticogenesis, Cyp19a1, neural development, neural stem cell, radial migration
The unique organization and architecture of the cerebral cortex is established during embryonic development. This organization is achieved in an “in-side-out” fashion, with inner, or deep layers forming first, and outer, or superficial layers developing last (Evsyukova, Plesant, & Anton, 2013). The development of the cortex and its laminar organization is controlled in large part by the coordinated processes of neurogenesis and cell migration. The process of radial neuronal migration is a multistep process (Evsyukova et al., 2013). Initially, newly born neurons, generated from neural stem cells, detach from the apical surface of the germinal ventricular zone (VZ). These neurons adopt a multipolar morphology and move from the VZ/subventricular zone (SVZ) into the intermediate zone (IZ; Noctor, Martinez-Cerdeno, Ivic, & Kriegstein, 2004). Here, neurons develop a bipolar shape and migrate along radial glia to their final position in the cortical plate (CP; Kawauchi, 2015). Once in this position, neurons can begin to form synaptic connections and thus contribute to circuit formation (Evsyukova et al., 2013).

Several areas of the brain have been shown to develop differently in males and females. In humans, during adolescent development, the brain undergoes differential development trajectories that lead to sex-specific differences in total cerebral volume and different local grey matter nuclei volumes (Kaczukurkin, Raznahan, & Satterthwaite, 2019). Notably, female total cerebral volume peaks earlier in adolescence than males (Lenroot et al., 2007). In rodents, analysis of neural circuitry and behaviour reveals that different systems and regions within the brain are engaged in a sex-dependent manner and specific nuclei within the brain are thought to be responsible for sex-specific behaviours (Choleris, Galea, Sohrabji, & Frick, 2018; Gillies & McArthur, 2010). For example, the hippocampus and basolateral nucleus of the amygdala are necessary for sex-dependent learning in rats (Bangasser & Shors, 2007; Waddell, Bangasser, & Shors, 2008). However, other regions of the rat brain also display sex-specific neural circuitry, including the bed nucleus of the stria terminalis (thalamus) and the medial prefrontal cortex (Bangasser & Shors, 2007; Maeng, Waddell, & Shors, 2010). Much of these differences are thought to be established during early neurodevelopment (Gillies & McArthur, 2010; McCarthy, Herold, & Stockman, 2018). However, an emerging theme is that even in the absence of an overt phenotypic difference in brain structure or circuitry, that males and females employ sex-specific mechanisms during development and within the adult brain (Choleris et al., 2018; Gillies & McArthur, 2010; Srivastava, Waters, et al., 2011). Such differences would not only influence our understanding of brain development in males and females, but also potentially have implications for our understanding of how neurodevelopmental disorders emerge in either sex (Choleris et al., 2018; Gillies & McArthur, 2010).

Oestrogens, in particular 17β-oestradiol (oestradiol), are integral in establishing sex differences during brain development in rodents, including the neural circuitry that underlies sex-typical and socio-aggressive behaviours (Choleris et al., 2018; Gillies & McArthur, 2010; McCarthy et al., 2018). Oestradiol stimulates the proliferation and differentiation of neural progenitors and neuronal populations in vitro (Bramvall, Korhonen, & Lindholm, 2002; Denley, Gafford, Sellers, & Srivastava, 2018; Okada et al., 2010). In organotypic cortical slices prepared from E15, neocortex exogenous oestradiol increases the proliferation of cortical progenitor cells just after 3 hr, and up to 3 days of treatment (Martinez-Cerdeno, Noctor, & Kriegstein, 2006). Oestradiol has also been shown to regulate neurite outgrowth in developing hippocampal neurons in a sex-specific manner. Exogenous oestradiol increased neurite outgrowth in male hippocampal neurons, but interestingly had no effect in developing female hippocampal neurons (Ruiz-Palmero et al., 2016). A similar effect has also been described in immature cortical neurons derived from male human-induced pluripotent stem cells (Shum et al., 2015). Interestingly, oestrogen receptor-beta (ERβ) knockout animals have been reported to display abnormal neuronal migration in the neocortex (Wang, Andersson, Warner, & Gustafsson, 2003). However, whether oestradiol influences neuronal migration in males and females is not known. Nevertheless, these lines of evidence indicate that oestrogens play a role in during the development of the cortex.

Aromatase, the enzyme responsible for the conversion of androgens to oestrogens is expressed across species. It is the rate-limiting step in the generation of oestradiol synthesized de novo within the brain. In the adult brain, aromatase is expressed in multiple regions, including the hippocampus and cortex (Lu et al., 2019; Saldanha, Remage-Healey, & Schlinger, 2011; Srivastava, Woolfrey, Liu, Brandon, & Penzes, 2010; Srivastava, Woolfrey, & Penzes, 2013). In vitro, hippocampal neurons have been shown to express aromatase and to locally synthesize oestriadiol (Fester et al., 2006; Kretz et al., 2004; Prange-Kiel, Wehrenberg, Jarry, & Rune, 2003). Similarly, in both rats and mice oestradiol has been measured in vivo in the cortex and hippocampus of male and female even in the absence of gonads (Hojo et al., 2004; Hojo & Kawato, 2018; Tuscher et al., 2016). Aromatase is expressed in male and female brains, and has been detected in the cortex (Beyer, Green, Barker, Huskisson, & Hutchison, 1994; Denley et al., 2018; MacLusky, Walters, Clark, & Toran-Alender, 1994; Martinez-Cerdeno et al., 2006; Yague et al., 2006, 2008). During prenatal development, aromatase activity has been measured within the cortex of rodents and non-human primates (MacLusky, Naftolin, & Goldman-Rakic, 1986; MacLusky et al., 1994; Martinez-Cerdeno...
et al., 2006). Similarly, oestradiol has been measured in both male and female cortices during prenatal development and just after birth (Konkle & McCarthy, 2011; MacLusky et al., 1986, 1994). Studies in zebrafish demonstrated that radial glial cells expressing aromatase divide into neurons and that neural stem cells in the ventricular layer also express aromatase (Pellegrini et al., 2007). In the developing mouse, aromatase is highly expressed in radial glia and intermediate progenitor (IP) cells (Martinez-Cerdeno et al., 2006). Moreover, inhibition of aromatase activity in E15 organotypic slices alters cortical progenitor proliferation (Martinez-Cerdeno et al., 2006). In vitro, aromatase was found to be expressed higher in male compared with female developing hippocampal neurons (Ruiz-Palmero et al., 2016). Interestingly, inhibition of aromatase had opposing effects on neurite outgrowth in male and female hippocampal neurons, indicating sex-specific actions of de novo synthesized oestriadiol (Ruiz-Palmero et al., 2016). Taken together, these data provide strong evidence for a role for aromatase in neuronal differentiation and neurogenesis. The purpose of this study was to investigate how the local synthesis of oestrogens affect neurodevelopment of the cerebral cortex and whether this differs between males and females. Using an shRNA approach, we knocked down the Cyp19a1 gene, which encodes aromatase, from an early developmental stage and assessed how the loss of aromatase impacted neural migration in the developing cortex. Analysis of neural migration revealed a sex-specific effect of aromatase loss on neural migration with male and female brains displaying opposing phenotypes. Taken together, these data contribute to the current evidence that brain-synthesized oestrogens play a role in the development of the cortex, and, moreover, add to the growing appreciation of sex-specific actions in the brain.

2 | MATERIALS AND METHODS

2.1 | Reagents

Antibodies used are as follows: GFP chicken polyclonal (ab13970; Abcam; 1:10,000); turboGFP (tGFP) rabbit polyclonal (PA5-22688, Thermo Fisher; 1:2,000); Myc mouse monoclonal (clone 2D11A8; 66004-1-lg; ProteinTech; 1:2,000); β-actin mouse monoclonal (clone 7D2C10; 60008-1-lg; ProteinTech); aromatase rabbit polyclonal (ab69653; Abcam; 1:100)—epitope is not described by the supplier; and aromatase rabbit polyclonal (ab18995; Abcam; 1:100). This antibody is raised against a peptide surrounding amino acid 385 of human aromatase, an area that is conserved across multiple species and has been reported to show similar staining to several other aromatase antibodies in rodent brain (Zhang et al., 2018) and specificity tested using ectopic expression of aromatase and an shRNA against the Cyp19a1 gene (Yan et al., 2019). A full list of antibodies used in this study can be found in Table 1. A myc-DDK-tagged murine aromatase construct (pCMV6-myc-DDK-aromatase) was purchased from OriGene (Rockville; Cat. No. MR224509);

| Antibody               | Host species         | Catalogue no. and company | Dilution used (ICC/IHC) | Dilution used (Western blotting) |
|------------------------|----------------------|---------------------------|-------------------------|----------------------------------|
| Aromatase              | Rabbit polyclonal    | ab18995 (Abcam)           | 1:200–1:100             | –                                |
| Aromatase              | Rabbit polyclonal    | ab69653 (Abcam)           | –                       | 1:100                            |
| Myc                    | Mouse monoclonal     | Clone 2D11A8; 66004-1-lg (ProteinTech) | 1:1,000                | 1:2,000                           |
| GFP                    | Chicken polyclonal   | ab13970 (Abcam)           | 1:10,000                | NA                               |
| TurboGFP (tGFP)        | Rabbit polyclonal    | PA5-22688 (Thermo Fisher) | –                      | 1:1,000                           |
| β-actin                | Mouse monoclonal     | clone 7D2C10; 60008-1-lg; ProteinTech | –                      |                                  |
| Anti-mouse HRP         | Goat                 | 31430 (Thermo Fisher)     | –                       | 1:10,000                          |
| Anti-rabbit HRP        | Goat                 | 31460 (Thermo Fisher)     | –                       | 1:10,000                          |
| Anti-chicken Alexa Fluor 488 | Goat              | A11039 (Thermo Fisher)   | 1:1,000                | –                                |
| Anti-rabbit Alexa Fluor 488 | Goat              | A11034 (Thermo Fisher)   | 1:750                   | –                                |
| Anti-mouse Alexa Fluor 568 | Goat              | A11031 (Thermo Fisher)   | 1:500                   | –                                |
| Anti-rabbit Alexa Fluor 647 | Goat              | Ab150083 (Abcam)          | 1:500                   | –                                |
the pCAG-eGFP has previously been described (Srivastava et al., 2012).

2.2 | Animals

Pregnant CD1 mice and Sprague-Dawley rats were obtained from Charles River. All experimental procedures were carried out in accordance with the Home Office Animals (Scientific procedures) Act, United Kingdom, 1986, or in accordance with animal procedures approved by Johns Hopkins Animal Care and Use committee complying with the standards of the National Institutes of Health. All animals were habituated for 3–5 days following standard housing conditions in accordance with institutional guidelines, following transport from vendor, before being used for experimentation.

2.3 | ShRNA constructs and validation

Four shRNAs against Musculus Cyp19a1 and one control scrambled shRNA were obtained from OriGene (Rockville; Cat. No. TG509276). These plasmids express both shRNA under the control of the U6 promoter and turboGFP (tGFP) under the control of a CMV promoter. The effectiveness of each shRNA was validated by testing the ability of each construct to knockdown myc-DDK-tagged murine aromatase expression in hEK293T cells. Briefly, hEK293 cells were grown to 40% confluency before transfection of myc-DDK-aromatase with or without shRNA constructs using Lipofectamine 2000 (Life Technologies). Transfections were allowed to proceed for 48 hr, after which cells were lysed and prepared for Western blotting or fixed and used for immunocytochemistry.

2.4 | Western blotting

Western blot analysis was performed on either CD1 mouse embryonic tissue or transfected hEK293 cells. Neocortical tissue from E7, 14, and P0 was harvested from CD1 mice. Tissue was weighed per sample and lysed 10 x v/w in ice-cold neuronal lysis buffer: 50 mM NaPO4; 40 mM NaCl, 5 mM EDTA; 5 mM EGTA; and 1% Triton X-100 + protease and phosphatase inhibitors. Lysates were then sonicated with 20 pulses before being centrifuged at 14,000 g for 20 min at 4°C to remove the nuclear fraction and large cell organelles. For hEK293 experiments, cells were harvested in lysis buffer: 50 mM Tris–HCl, pH 7.4; 1 mM EDTA; 1 mM EGTA; 150 mM NaCl; and 1% Triton X-100, + protease inhibitors. Lysates were passed through a 21-gauge needle 15 times before being centrifuged to remove the nuclear fraction and large cell organelles. Sample buffer was added to all lysates, which were then denatured for 5 min at 95°C and stored at −80°C until used further. All lysates were separated on 10% in-house made acrylamide gels through electrophoresis and transferred onto polyvinylidene difluoride (PVDF) at 78 mA for 990 min at 4°C. Membranes were blocked for 1 hr in TBS-T (with 0.1% Tween) containing 5% bovine serum albumin (BSA; Sigma: A7906) followed by an overnight incubation of specific primary antibodies (Table 1) in the same blocking solution at 4°C with agitation. This was followed by secondary HRP (Table 1) incubation in the blocking solution for 2 hr at room temperature. Membranes were then incubated in Clarity Western ECL substrate (Bio-Rad: 170-5061) for 5 min before protein detection using the ChemiDoc XRS+ imaging system (Bio-Rad: 170-8265) running ImageLab™ software version 5.2.1 (http://www.bio-rad.com/en-uk/products/image-lab-software; Bio-Rad). Quantification of bands was performed by measuring the integrated intensity of each band and normalizing to β-actin, for protein loading, using ImageJ. Quantified Western blot data were processed post hoc in MS Excel to eliminate batch effects (Degasperi et al., 2014). Briefly, the sum of all conditions in each data set was calculated. Each condition was then divided by the sum of all conditions to remove batch effects. Data were then log-transformed and plotted.

2.5 | Neuronal culture and transfections

Mixed-sex cortical neuronal cultures were prepared from E18 Sprague-Dawley rat embryos as described previously (Srivastava, Woolfrey, & Penzes, 2011). Animals were habituated for 3 days before experimental procedures—a total of 3 pregnant dams were used to generate mixed-sex primary cortical neurons. Cells were plated onto 18-mm glass coverslips (No 1.5; 0117580, Marienfeld-Superior GmbH & Co.), coated with poly-d-lysine (0.2 mg/ml, Sigma), at a density of 3 x 10^5/well equal to 857/mm². Neurons were cultured in feeding media: neurobasal medium (21103049) supplemented with 2% B27 (17504044), 0.5 mM glutamine (25030024) and 1% penicillin:streptomycin (15070063; all reagents from Life Technologies). Primary cortical neurons were transfected with scramble, negative or aromatase-shRNA one day after plating (days in vitro (DIV) 1) using Lipofectamine 2000 (11668027, Life Technologies). Briefly, 4–6 µg of plasmid DNA was mixed with Lipofectamine 2000 and incubated for 4–12 hr, before being replaced with fresh feeding media. Transfections were allowed to proceed for 4 days (DIV 5) after which cells were fixed and processed for immunocytochemistry (ICC).

2.6 | In utero electroporation

In utero electroporation targeting the somatosensory cortex was performed according to previously published protocol (Niwa et al., 2010; Saito et al., 2016). Embryos were electroporated
at E14.5. Pregnant CD1 mice (obtained from Charles River) were anaesthetized by intraperitoneal administration of a mixed solution of ketamine HCl (100 mg/kg), xylazine HCl (7.5 mg/kg) and buprenorphine HCl (0.05 mg/kg). The uterine sacs were exposed by laparotomy. In each pregnant animal, aromatase-shRNA, scramble-shRNA (1 μg/μl) or eGFP (0.5 μg) was injected into the left ventricle of the embryo with a glass micropipette made from a microcapillary tube (GD-1; Narishige). Control embryos were injected eGFP (1.0 μg/μl) into the right ventricle in the same manner. The head of the embryo was held between the electrodes (Nepagene) placed over the posterior forebrain with the positive electrode positioned above sight of injection. Electrode pulses (33 V; 50 ms) were charged four times at intervals of 950 ms with an electroporator (CUY21EDIT; Nepagene). After electroporation, the uterine horn was replaced in the abdominal cavity to allow the embryos to continue to develop. A total of 6–15 embryos were electroporated in each of the 5 pregnant animals. Brains were extracted from P0 pups and assessed for GFP expression as described below. The embryos to continue to develop. A total of 6–15 embryos were electroporated in each of the 5 pregnant animals. Brains were extracted from P0 pups and assessed for GFP expression as correct location prior to fixation. Brains were fixed by overnight immersion in 4% paraformaldehyde or 4% sucrose PBS for 20 min at room temperature. Fixed neurons were then permeabilized and blocked simultaneously (2% normal goat serum, 5425S, New England Biolabs, and 0.1% Triton X-100) before incubation in primary antibodies overnight and subsequent incubation with secondary antibodies the following day (Table 1).

Brains were mounted in OCT embedding media (Bright) and cut into 14-μm sections across the coronal plane using a cryostat (Leica CM 1860 UV, Ag Protect) and collected on SuperFrost Plus microscope slides (Thermo Scientific). Immunohistochemistry (IHC) was carried out as previously described (Jones, Sumiya, Woolfrey, Srivastava, & Penzes, 2019). In brief, sections were simultaneously permeabilized and blocked in 0.1% Triton X-100 with 2% normal goat serum in PBS for 1 hr at room temperature, in a humidified chamber. They were then incubated overnight at 4°C in a humidified chamber with primary antibodies against chicken GFP (1:1,000; Abcam) and rabbit aromatase (1:100 Abcam; Table 1). Sections were then counterstained with the appropriate secondary antibodies and counterstained with DAPI (Thermo Fisher D1306). Images were captured and analysed as described below.

2.9 Image acquisition and data analysis

Confocal images of IHC-stained P0 sections were carried out using a Nikon spinning disc confocal equipped with either a 20x or 40x objective. Image z-stacks were acquired at z-intervals optimized for the specific objective. Images to be used for subsequent intensity-based analysis were acquired using identical acquisition parameters. Analysis of aromatase expression was carried out on sections immunostained for aromatase, GFP and DAPI. Regions of interest (ROIs – 300 x 300 pixels) were determined by GFP staining. Following acquisition, images were background subtracted, and the mean intensity of aromatase staining determined for five independent ROIs per image; three ROIs of background staining were also measured for each section. The mean intensity for each section was normalized to background staining (average of 3x background ROIs + 2x StDev). Between 3 and 4 sections per brain, 4–5 brains per condition were used for these analyses. Where multiple sections from a single brain were used, data were averaged to a single data point per brain to avoid pseudo-replication.

For migration analysis, cortical subsections were identified by DAPI staining and ROIs were identified by GFP and DAPI staining. The effect of aromatase knockdown on migration was analysed by quantitative bin analysis according to previously published methods (Kubo et al., 2010). In brief, the cortex was divided into ten equal sections (bins) and percentage of GFP+ cells within each bin was determined. For each condition, 3–4 independent in utero electroporations were performed. Between 6 and 11 brains across...
all electroporation were imaged. Up to 3 sections per brain were imaged and analysed. Where multiple sections from a single brain were used, data were averaged to a single data point per brain to avoid pseudo-replication. The numbers of uni/bipolar (i.e. one to two primary processes), multipolar (i.e.>two primary processes) or round cells (i.e. no observable processes) were quantified in the VZ/SVZ, IZ and CP in at least 5 brains per condition, per sex.

Images of fixed and immunostained primary cultures were acquired with a Zeiss Axio Imager Z1 using a 40x oil-immersion objective (Carl Zeiss, N.A., 1.2). Two-dimensional maximum projection reconstructions of images were generated, and linear density calculated using ImageJ/Fiji (https://imagej.nih.gov/ij/). Mean grey value of aromatase immunofluorescence was measured automatically using ImageJ. Cultures directly compared were stained simultaneously and imaged with the same acquisition parameters. In the green/magenta colour scheme, co-localization is indicated by white overlap.

### 2.10 Statistical analysis

All datasets were tested for normality using the D’Agostino & Pearson normality test prior to inferential statistical analyses (D’Agostino & Belanger, 1990). Data sets found to be normally distributed were analysed using parametric statistical tests, whilst data sets found to be abnormally distributed were analysed using their non-parametric equivalent. Two-tailed unpaired Student’s t test used for comparisons between 2 groups. Ordinary one-way analysis of variance (ANOVA) with Bonferroni’s correction for multiple comparisons was used to examine data sets with a single variable. Two-way ANOVA with Bonferroni’s correction for multiple comparisons was used for data sets with two factors. All data visualizations were generated in GraphPad Prism 8.0 (GraphPad Software, http://www.graphpad.com/scientific-software/prism/). All data are shown as mean ± standard error of the mean (SEM), and all error bars represent SEM.

### 3 RESULTS

#### 3.1 Sex differences in aromatase expression but not in migration during cortex development

Previous studies have observed expression of aromatase in mouse embryonic neocortical tissue from E9 throughout to E15 in radial glia and IP cells (Martinez-Cerdeno et al., 2006). To assess whether aromatase was also expressed at later stages of development, we first probed Western blots of murine CD1 neocortical tissue derived from E7, 14, 17 and P0 time points with an antibody raised against a synthetic peptide based on human aromatase. A band of around 57 kDa could be observed in E7, 14, 17 and P0 neocortical mouse tissue (Figure 1a). However, in addition to the prominent band around 57 kDa, additional bands could also be readily detected. These bands indicate that this antibody had non-specific binding in Western blotting. Considering the non-specificity of the aromatase antibody used in our Western blot analysis, we tested a second aromatase antibody for use in immunostaining experiments. This antibody detects a conserved region surrounding amino acid 358 of human aromatase. The antibody has recently been used for immunohistochemical studies and demonstrated comparable staining to two different and well-published aromatase antibodies in rats (Zhang et al., 2018) and has further been tested for specificity using a knockdown approach in mouse NSC-34 motor neuron-like cells (Yan et al., 2019). To confirm specificity of this antibody, hEK293 cells were transfected with a myc-DDK-tagged murine aromatase construct and exogenous aromatase expression detected using antibodies against either myc or aromatase (Figure 1b). Exogenous aromatase localized throughout the cell including at the end of processes. Myc and aromatase immunofluorescence could readily be observed within the same cell. Closer examination of myc and aromatase immunostaining using line-scan analysis of fluorescence intensity demonstrated that myc and aromatase staining co-localized (Figure 1b). No immunofluorescence could be observed in non-transfected cells. Furthermore, immunostaining of adult mouse brain using this antibody produced comparable staining to previous reports (Foidart, Harada, & Balthazart, 1995). Strong aromatase immunoreactivity was observed in the ventromedial hypothalamus and arcuate hypothalamic nucleus, with weaker staining in the paraventricular thalamic nuclei, habenular nucleus, lateral posterior thalamic nuclei and primary somatosensory cortex (Figure S1). These data indicate that this aromatase antibody is able to detect mouse aromatase. However, it is of note that without validation using knockout tissue that there is always uncertainly with regard to the specificity of any antibody.

Using primary embryonic hippocampal neurons, Ruiz-Palmero and colleagues have reported higher aromatase expression in developing male neurons compared with female neurons (Ruiz-Palmero et al., 2016). In order to establish whether a sex difference in aromatase expression could also be detected in the developing cortex, we examined the expression of this enzyme in male and female P0 mice using immunohistochemistry. Sex of P0 mice was established by the assessment of sex-determining region Y (sry) gene expression (Figure 1c). In P0 male and female mice, aromatase-positive cells could be observed in the VZ/SVZ, IZ and CP regions of the cortex (Figure 1d). In line with previous studies (Montelli et al., 2012; Ruiz-Palmero et al., 2016), quantification of aromatase staining demonstrated
(a) 100 75 50 37
(b) DAPI Myc Aromatase
   Intensity (a.u.)
   Position
   Aromatase  Myc
(c) Sry
(d) Female P0  Male P0
   DAPI Aromatase
   CP IZ SVZ/VZ
   i) Aromatase
   ii) Aromatase
   Relative aromatase expression (a.u.)
   Female  Male
   ***
(f) Male P0
   i) DAPI Myc Aromatase
   CP
   ii) YZ XZ
   Aromatase
   GFP
   XZ
higher expression in P0 cortex of male compared with female P0 mice (aromatase staining intensity arbitrary units (a.u.): Student’s t test; t(26) = 6.22, two-tailed; n = 13–15 brains for each sex; Figure 1e). To determine whether aromatase was present in young migrating neurons, we labelled neural progenitor cells by in utero electroporation of embryos at E14.5 with a GFP-expressing plasmid (pCAG-eGFP); this method allows for the labelling of migrating neurons at later stages of development (Kubo et al., 2010; Niwa et al., 2010; Srivastava et al., 2012). At P0, GFP-positive (GFP+) migrating neurons were also positive for aromatase. Aromatase staining could be seen in both cell bodies as well as along processes of migrating cells; overlap of GFP and aromatase signals. Error bars represent SEM; each data point represents data from a single brain. Scale bars = 100 μm (d) and 20 μm (d inset and f) [Colour figure can be viewed at wileyonlinelibrary.com]
**FIGURE 3** Optimization of Cyp19a1 knockdown in vitro. (a) Western blot of cell lysates taken from hEK293 cells expressing myc-DDK-aromatase in the presence of shRNA against Cyp19a1 (shRNA_d) or a control construct (shRNA_scram). (b) Quantification of aromatase expression reveals that shRNA_c (aka shRNA_arom) produced ~60% knockdown of exogenous aromatase: \( *p < .05 \). (c) Representative confocal images of hEK293 cells expressing myc-DDK-aromatase in the presence of shRNA against Cyp19a1 (shRNA_arom), negative shRNA (shRNA_neg) or aromatase scramble-shRNA construct (control). In both control and shRNA_neg conditions, myc immunofluorescence was readily evidenced in GFP+ cells. However, in cells expressing shRNA_arom, almost no myc immunofluorescence could be detected. Quantification of myc intensity to determine exogenous aromatase expression revealed that shRNA_arom-expressing cells had significantly reduced expression of exogenous construct: \( ***p < .001 \). \( ***p < .001 \). (d) Representative images of DIV 5 mixed-sex primary rat cortical neurons expressing scramble (control)-negative (shRNA_neg) or aromatase (shRNA_arom)-shRNA constructs. Neurons were immunostained for GFP and endogenous aromatase. Yellow arrowheads indicate cell body of GFP-expressing cells, whereas white arrowheads highlight GFP-negative cells. Quantification of endogenous aromatase expression in DIV 5 cortical neurons expressing control, shRNA_neg or shRNA_arom constructs: \( ***p < .001 \). Error bars represent SEM; each data point represents data from a single experiment (b) or individual cell (c and d). Scale bar = 10 μm [Colour figure can be viewed at wileyonlinelibrary.com]

Migration differed between male and female mice, we quantified the distribution of GFP-positive (GFP+) cells labelled by in utero electroporation, in P0 brains as previously described (Kubo et al., 2010). Analysis of neuronal migration revealed that there was no difference in the migration of GFP+ cells between either sex (Figure 2a). This observation is confirmed by the relatively parity of GFP+ cells throughout the developing P0 cortex (percentage GFP+ cells per binned region: two-way ANOVA; interaction of sex and binned region, \( F(9, 150) = 0.3938, p = .9366 \); main effect of sex, \( F(1, 150) = 3.7e-19, p > .999 \); \( n = 9–11 \) brains per sex from 3 to 4 independent electroporations; Figure 2b). We further assessed the morphology of migrating cells within the VZ/SVZ, IZ and CP regions of the cortex. Specifically, we determined the number of GFP+ cells that had a multipolar (nascent neurons), uni/bipolar (migrating neurons) or round (unclassified) morphology (Figure 2c). Once more, no difference was observed between sex, with a similar number of GFP+ cells in each morphological class (percentage GFP-positive cells of each morphology: two-way ANOVA; interaction of sex and cell morphology, (VZ/SVZ), \( F(2, 24) = 1.026, p = .3736 \); main effect of sex (VZ/SVZ), \( F(1, 24) = 2.5e-18, p > .999 \); interaction of sex and cell morphology (IZ), \( F(2, 24) = 0.082, p = .9218 \); main effect of sex (IZ), \( F(1, 24) = 1.7e-18, p > .999 \); interaction of sex and cell morphology (CP), \( F(2, 24) = 1.403, p = .2653 \); main effect of sex (CP), \( F(1, 24) = 0.001, p > .999 \); \( n = 5 \) brains per sex from 3 independent electroporations; Figure 2d). Taken together, these data indicate that there are no differences in neuronal migration between male and female mice at P0.

### 3.2 Validation of aromatase knockdown

Although our data indicate that there is no difference in neuronal migration between males and females under control conditions, previous studies using ERβ knockout animals have suggested a role for oestradiol in radial migration (Wang et al., 2003). Furthermore, oestradiol has been shown to regulate proliferation of neural progenitor cells (Brannvall et al., 2002; Martinez-Cerdeno et al., 2006), and to have sex-specific effects on neurite outgrowth of developing hippocampal neurons (Ruiz-Palmero et al., 2016). Therefore, we were interested in understanding whether brain-derived oestrogens may contribute to neuronal migration in either male or female mice. In order to do this, we employed a short hairpin RNA interference (shRNA) approach to selectively knockdown expression of Cyp19a1, the gene encoding aromatase. The efficiency of four individual shRNA to knockdown aromatase was first established in hEK293 cells. Myc-DDK-tagged murine aromatase (myc-DDK-aromatase) was exogenously expressed in hEK293 cells in the presence of either a shRNA for aromatase or a control (scramble) shRNA; knockdown efficiency was determined by measuring the intensity of the myc signal (Figure 3a). Of the four shRNA tested, shRNA_c (herein referred to as shRNA_arom) reduced myc-aromatase expression by ~60% (relative aromatase expression arbitrary units (a.u.): one-way ANOVA; \( F(5, 12) = 6.65, p = .0035 \); Bonferroni’s post hoc test; \( n = 3 \) independent experiments; Figure 3a,b). We repeated this experiment using an ICC approach. HEK293 cells exogenously expressing murine myc-DDK-aromatase were co-transfected with control (scramble) shRNA, shRNA_arom or a shRNA which did not show any ability to knockdown exogenous aromatase in hEK293 cells (shRNA_d herein referred to ask shRNA_neg; Figure 3c). Consistent with our Western blotting data, the shRNA_arom construct reduced aromatase staining, as determined by myc immunostaining, by 45% compared with the control shRNA. The shRNA_neg construct had a negligible effect on myc expression (relative aromatase expression arbitrary units (a.u.): one-way ANOVA; \( F(2, 87) = 21.99, p < .001 \); Bonferroni’s post hoc test; \( n = 30 \) cells from 3 independent experiments; Figure 3c).

We further validated knockdown of aromatase by testing the shRNA_arom constructs in developing mixed-sex rat primary cortical neurons. Primary neurons were transfected with a scramble (control) shRNA, shRNA_neg or shRNA_arom (Figure 3d). In control and shRNA_neg conditions, endogenous aromatase could be clearly observed in GFP-positive and GFP-negative cells. Aromatase was present within the
cells body as well as along processes (Figure 3d). Conversely, in shRNA_arom-expressing cells, endogenous aromatase expression was lower than GFP-negative cells. Quantification of aromatase expression in GFP-positive cells confirmed these observations (relative aromatase expression arbitrary units (a.u.): one-way ANOVA; F(2, 44) = 28.13, p < .0001; Bonferroni’s post hoc test; n = 14–17 cells from 3 independent cultures; Figure 3d). It is of note that validation of these shRNA constructs could be performed functionally by measuring production of oestradiol in primary cells expressing shRNA constructs. Such experiments are beyond the scope of the current manuscript but should be performed in future studies. Nevertheless, these data provide evidence that the shRNA_arom was able to knockdown expression of exogenous murine aromatase as well as of endogenous rodent aromatase in developing rodent neurons.

3.3 Validation of aromatase knockdown in vivo

Next, we assessed whether expression of shRNA_arom could knockdown aromatase in vivo. To this end, we used in utero electroporation to express shRNA_scram (control) or shRNA_arom expression constructs in the developing cortex of CD1 male and female mice. Embryos were electroporated at E14.5, brains collected at P0. Samples were genotyped for sex, and cortical sections underwent IHC for GFP and aromatase (Figure 4). Confocal images of P0 neocortex revealed that GFP+ cells in shRNA_arom conditions had reduced aromatase expression in male and female pups (Figure 4a,b). This could further confirm by orthogonal projections showing the presence of aromatase staining in control or shRNA_arom conditions—overlap is indicated in white in this colour scheme. (c) Quantification of aromatase expression in GFP+ cells in control or shRNA_arom conditions in female or male P0 cortex. This revealed that shRNA-arom reduced endogenous aromatase expression by 53%–55% in either sex; ***p < .001. Error bars represent SEM; each data point represents data from a single brain. Scale bars = 20 μm [Colour figure can be viewed at wileyonlinelibrary.com]

Taken together, these data confirm the efficacy of aromatase knockdown by shRNA in vivo.

3.4 Aromatase knockdown affects cortical migration divergently in males and females

To determine whether brain-derived oestrogens play a role in the migration of neocortical cells, we assessed the distribution of GFP+ cells in control and aromatase knockdown conditions in P0 mice. Knockdown of aromatase in male mice resulted in an increase in the number of GFP+ cells within the upper most portion of the CP and a reduction in GFP+ cells within the SVZ/VZ compared with male mice electroporated with a control shRNA (percentage GFP+ cells per binned region: two-way ANOVA; interaction of binned region and condition (shRNA construct), F(9, 160) = 10.08, p < .0001, main effect of binned region, F(9, 160) = 127.3, p < .0001, main effect of shRNA constructs, F(1, 160) = 2.4e-15, p > .999; Bonferroni’s post hoc; n = 9–11 brains per condition from 3 to 4 independent electroporations; Figure 5a,b). Examination of female P0 mice revealed an opposite phenotype (Figure 5c). Female shRNA_arom-expressing mice had a decrease in the number of GFP+ cells within the CP and a concurrent increase in the number of GFP+ cells within the SVZ/VZ compared with control condition (percentage GFP+ cells per binned region: two-way ANOVA; interaction of binned region and condition (shRNA construct), F(9, 140) = 5.873, p < .0001, main effect of binned region, F(9, 140) = 149.2, p < .0001, main effect of shRNA constructs, F(1, 140) = 0.15, p = .698; Bonferroni’s post hoc; n = 6–10 brains per condition from 3 to 4 independent electroporations; Figure 5c,d).

Finally, we examined whether knockdown of aromatase altered the proportion of uni/bipolar, multipolar and round cells within each cortical region in each sex. Comparison of control and shRNA_arom conditions in male P0 mice did not reveal any differences in the distribution of cell morphologies in any cortical subregion (percentage GFP-positive cells of each morphology: two-way ANOVA; interaction of condition (shRNA construct) and cell morphology (VZ/SVZ), F(2, 24) = 0.1007, p = .9046, main effect of condition (VZ/SVZ), F(1, 24) = 1.39e-20, p > .999; interaction of condition (shRNA construct) and cell morphology (IZ), F(2, 24) = 0.117, p = .89, main effect of...
condition (IZ), $F(1, 24) = 18.14e-19, p > .999$; interaction of condition (shRNA construct) and cell morphology (CP), $F(2, 24) = .679, p = .5164$, main effect of condition (CP), $F(1, 24) = 1.57e-16, p > .999$; $n = 5$ brains per condition from 3 independent electroporations; Figure 6a). However, shRNA_arom P0 female mice had an increase in the number of round morphology cells and decrease in unipolar/bipolar cells, consistent with a reduction in the number of migrating cells (percentage GFP-positive cells of each morphology: two-way ANOVA; interaction of condition (shRNA construct) and cell morphology (VZ/SVZ), $F(2, 24) = 30.62, p < .0001$, main effect of condition (VZ/SVZ), $F(1, 24) = 1.12e-19, p > .999$; interaction of condition (shRNA construct) and cell morphology (IZ), $F(2, 24) = 5.777, p = .009$, main effect of condition (IZ), $F(1, 24) = 9.85e-18, p > .999$; interaction of condition (shRNA construct) and cell morphology (CP), $F(2, 24) = 0.666, p = .9365$, main effect of condition (CP), $F(1, 24) = 1.18e-17, p > .999$; Bonferroni’s post hoc, $n = 5$ brains per condition from 3 independent electroporations; Figure 6b). Taken together, these data indicate that that knockdown of aromatase may accelerate radial neuronal migration in male, whereas migration is impaired in the female developing cortex.

4 | DISCUSSION

Aromatase is expressed in specific brain regions, where it controls the bioavailability of brain-synthesized oestradiol within female and male brains (Lu et al., 2019; Saldanha et al., 2011; Srivastava et al., 2013). Moreover, oestradiol is present at significant levels in the brain of both sexes, including the developing cortex (Konkle & McCarthy, 2011; MacLusky et al., 1986, 1994). However, the functions of brain-derived oestradiol during early corticogenesis development are unclear (Denley et al., 2018). Here, we demonstrated that aromatase is expressed within the developing cortex of prenatal female and male mice. Aromatase expression was higher in males compared with females across the different laminae of the developing cortex. Interestingly, there were no differences in the pattern of migration in female and male cortices at P0. We used a knockdown approach to suppress endogenous Cyp19a1, and thus, reduce aromatase expression in a subset of cortical progenitor cells destined to migrate to layer 2/3. Knockdown of aromatase had opposing effects on the migration of cortical progenitor cells in female and male developing brains. Specifically, loss of aromatase in male mice resulted in an increase in GFP+ cells in the CP with a concurrent decrease in the SVZ/VZ, indicating a potential increase in radial migration. Conversely, knockdown of aromatase in female mice resulted in a significantly decreased number of GFP+ cells in the upper layers of the developing cortex and an accumulation of cells within the SVZ/VZ, indicating a potential decrease in neuronal migration. Taken together, our data indicate that whilst there is no obvious difference in migration between males and females under control conditions, the influence of brain-derived oestradiol on radial migration is sex-specific and suggests that brain-derived oestrogens may regulate neuronal migration via sex-specific mechanisms.

The data presented in this study are consistent with a purported role for brain-derived oestradiol in the development of the mammalian forebrain. Previous studies have demonstrated that aromatase is expressed in multiple regions of the brain, including the cortex (Beyer et al., 1994; MacLusky et al., 1994; Martinez-Cerdeno et al., 2006; Yague et al., 2008). In vitro, aromatase expression has been reported to be higher in males than in female hippocampal neurons (Ruiz-Palmero et al., 2016). Our immunostaining data are consistent with these findings, in that aromatase expression was also found to be higher in male compared with female P0 brains. It is of note, however, that antibodies against aromatase have varying efficiencies. We have used an antibody previously shown to give comparable staining to two independent antibodies (Zhang et al., 2018), as well as being validated by shRNA-mediated knockdown in a previous (Yan et al., 2019) and in the current study. Nevertheless, future studies using additional antibodies as well as independent approach are needed to validate these findings. Previous work has implicated ERβ knock-out in the development of the cortex (Wang et al., 2003). Furthermore, aromatase knockout mouse display disorganization of the developing cortex (Anthoni et al., 2012). In the current study, we provide evidence that brain-derived oestradiol regulates neuronal migration in the developing cortex of both female and male mice. Whether and how systemic oestradiol also impacts neuronal migration are unclear from these studies and would need to be studied further in the future.

A striking finding of this study is that the effect of aromatase knockdown on neuronal migration in the developing cortex is opposing in female and male mice. Interestingly, de novo synthesized oestradiol has sex-specific effects on neurite outgrowth in developing hippocampal neurons (Ruiz-Palmero et al., 2016), reflecting observations in our study. There are two possible explanations for the observed sex-specific effects of aromatase knockdown. First, oestradiol could be exerting multiple effects on progenitor cells, such as controlling cell proliferation and/or apoptosis, as reported previously in the developing cortex, hippocampus and hypothalamus (Brannvall et al., 2002; Denley et al., 2018; Fester et al., 2006; Martinez-Cerdeno et al., 2006; McCarthy et al., 2018). Indeed, previous studies have shown that exogenous oestradiol application both ex vivo on organotypic slices generated from E15 cortex
and in vivo resulted in an increase in cortical progenitor proliferation (Martinez-Cerdeno et al., 2006). However, this study did not report if these effects were seen in both male and female developing cortices. A second possibility is that oestradiol could be modulating the migration of newly born neurons in both sexes. In this study, no differences in the number of GFP+ cells was measured in the uppermost bins, equating to the upper portion of the CP, *p < .05, ***p < .001. (c) Representative confocal images of male P0 cortex in utero electroporated at E14.5 with either control (shRNA scram) or shRNA_arom and stained for GFP. (d) Quantification of GFP+ cell distribution throughout the developing male cortex. Loss of aromatase induced by Cyp19a1 knockdown (shRNA_arom) resulted in a significant increase in GFP+ cells in the upper portion of the CP; *p < .05. Congruent with this, a decrease in GFP+ cells was observed in the SVZ/VZ of the P0 male cortex. Scale bars = 50 μm [Colour figure can be viewed at wileyonlinelibrary.com]
was true on endogenous application of oestradiol (Bender et al., 2010). Thus, it is possible that loss of aromatase within the developing cortex could impact reelin expression and therefore contribute to the observed phenotypes albeit via differential mechanisms within male and female developing brains.

It is also important to note that although using a knockdown approach in combination with in utero electroporation allows us to examine the impact of aromatase knockdown on GFP+ cells, it is unclear whether non-GFP+ cells are also impacted. Oestradiol can act in a paracrine and autocrine fashion (Prange-Kiel et al., 2003). In addition, it is important to note that off-target effects of shRNA may be confounded in the results. Thus, it would be important for future studies to understand whether the observed effects are due to a paracrine effect of depleted brain-derived oestradiol. As aromatase (Cyp19a1) haploinsufficiency has been reported to result in cortical disorganization in E17 pups (Anthoni et al., 2012), this model could be used to elucidate aromatase-mediated mechanisms underpinning developmental phenotypes such as neuronal migration.

The development of the cortex is fundamental for normal brain function. Interestingly, multiple animal models for autism spectrum disorders aimed at understanding the contribution of genetic and/or environmental risk factors to the underlying pathophysiology of this disorder have revealed that disruptions in early brain development are prevalent in these models. In particular, abnormalities in the development, migration and organization of the developing cortex have been reported (Fenlon et al., 2015; Varghese et al., 2017). Moreover, there is accumulating evidence that elevated levels of foetal steroids, especially testosterone (Baron-Cohen et al., 2015; McCarthy & Wright, 2017), are linked with autism spectrum disorders. Furthermore, rare mutations in the CYP191A gene have been reported in autistic patients and reduced ERβ and aromatase expression has been measured in autistic post-mortem tissue (Chakrabarti et al., 2009; Crider, Thakkar, Ahmed, & Pillai, 2014; Sarachana, Xu, Wu, & Hu, 2011). These lines of evidence have led to suggestions that altered steroidogenic activity and/or elevated levels of foetal testosterone could contribute to the pathophysiology of autism. It should be noted that

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**FIGURE 6** Knockdown of aromatase alters the number of uni/bipolar cells in female developing cortex. (a) Quantification of GFP+ cells with specific cellular morphology in VZ/SVZ, IZ and CP cortical subregions of control and shRNA_arom male mice. No difference was observed between conditions. (b) Quantification of GFP+ cells with specific cellular morphology in VZ/SVZ, IZ and CP cortical subregions of control and shRNA_arom female mice. In the VZ/SVZ subregion, an increase in cells with a round morphology and decrease in cells with uni/bipolar morphologies were observed; ***p < .001. Error bars represent SEM; each data point represents data from a single brain [Colour figure can be viewed at wileyonlinelibrary.com]
knocking down Cyp19a1 will both reduce oestradiol levels and increase the levels of testosterone and its metabolites dihydrotestosterone (DHT) and 5α-androstan-3β,17β-diol (3β-DIOL). Interestingly, both testosterone and DHT have sex-specific actions on neurite outgrowth (Ruiz-Palmero et al., 2016), actions thought to be dependent on signalling via the androgen receptor. Conversely, 3β-DIOL is known to also signal via ERβ (Srivastava et al., 2013). Therefore, the current study may not only provide an insight into how reduced brain-derived oestradiol levels impact development of the cortex, but may also provide insight into the impact that elevated levels of foetal testosterone may have on corticogenesis and with the knowledge that this effect would be limited in effect and brain region impacted in this model, and therefore how dysregulation of foetal steroids could contribute to the emergence of neurodevelopmental disorders such as autism spectrum disorders.

In conclusion, the current study revealed that aromatase is expressed in the developing cortex of both female and male mice, and at higher levels in males than females. Knockdown of aromatase in cortical progenitor cells destined to migrate to layer 2/3 had marked sex-specific effects. Future studies focusing on understanding the mechanism underlying these effects, including investigating the potential role of Rap1, and also identifying the receptors that are responsible for the actions of the brain-synthesized oestrogens (i.e. do brain-synthesized oestrogens function via the classical “genomic” mode of action or do they act via a “membrane initiated” mode of action), are required. Together with the current work, these studies will help reveal the potential role of foetal steroids in normal development and how perturbations in this system may contribute to the emergence of disease.

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CONFLICT OF INTEREST
The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

AUTHOR CONTRIBUTIONS
K.J.S., A.S., A.K. and D.P.S. designed experiments. K.J.S. M.C.S.D., A.S. E.M.F. and D.P.S. performed all experiments and subsequent analysis. D.P.S. oversaw the study; K.J.S. M.C.S.D., and D.P.S. wrote the manuscript; and all authors edited the manuscript drafts.

DATA AVAILABILITY STATEMENT
Primary data material can be accessed by contacting the corresponding authors.

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REFERENCES
Anthoni, H., Sucheston, L. E., Lewis, B. A., Tapia-Paez, I., Fan, X., Zucchelli, M., … Kere, J. (2012). The aromatase gene CYP19A1: Several genetic and functional lines of evidence supporting a role in reading, speech and language. Behavior Genetics, 42, 509–527. https://doi.org/10.1007/s10519-012-9532-3
Bangasser, D. A., & Shors, T. J. (2007). The hippocampus is necessary for enhancements and impairments of learning following stress. Nature Neuroscience, 10, 1401–1403. https://doi.org/10.1038/nn1973
Baron-Cohen, S., Auyeung, B., Norgaard-Pedersen, B., Houghard, D. M., Abdallah, M. W., Melgaard, L., … Lombardo, M. V. (2015). Elevated fetal steroidogenic activity in autism. Molecular Psychiatry, 20, 369–376. https://doi.org/10.1038/mp.2014.48
Bender, R. A., Zhou, L., Wilkars, W., Fester, L., Lanowski, J. S., Paysen, D., … Ruse, G. M. (2010). Roles of 17α-estradiol involve regulation of reelin expression and synaptogenesis in the dentate gyrus. Cerebral Cortex, 20, 2985–2995.
Beyer, C., Green, S. J., Barker, P. J., Huskisson, N. S., & Hutchison, J. B. (1994). Aromatase-immunoreactivity is localised specifically in neurones in the developing mouse hypothalamus and cortex. Brain Research, 638, 203–210. https://doi.org/10.1016/0006-8993(94)90651-3
Brannvall, K., Korhonen, L., & Lindholm, D. (2002). Estrogen-receptor-dependent regulation of neural stem cell proliferation and differentiation. Molecular and Cellular Neurosciences, 21, 512–520. https://doi.org/10.1006/mcne.2002.1194
Chakrabarti, B., Dudbridge, F., Kent, L., Wheelwright, S., Hill-Cawthorne, G., Allison, C., … Baron-Cohen, S. (2009). Genes related to sex steroids, neural growth, and social-emotional behavior are associated with autistic traits, empathy, and Asperger syndrome. Autism Research, 2, 157–177. https://doi.org/10.1002/aur.80
Choleris, E., Galea, L. A. M., Sohrabji, F., & Frick, K. M. (2018). Sex differences in the brain: Implications for behavioral and biomedical research. Neuroscience and Biobehavioral Reviews, 85, 126–145. https://doi.org/10.1016/j.neubiorev.2017.07.005
Crider, A., Thakkar, R., Ahmed, A. O., & Pillai, A. (2014). Dysregulation of estrogen receptor beta (ERβ), aromatase (CYP19A1), and ER co-activators in the middle frontal gyrus of autism spectrum disorder subjects. Molecular Autism, 5, 46. https://doi.org/10.1186/2040-2392-5-46
D’Agostino, R. B., & Belanger, A. (1990). A suggestion for using powerful and informative tests of normality. The American Statistician, 44, 316–321.

Degasperi, A., Birtwistle, M. R., Volinsky, N., Rauch, J., Kolch, W., & Khodolenko, B. N. (2014). Evaluating strategies to normalise biological replicates of Western blot data. PLoS ONE, 9, e87293. https://doi.org/10.1371/journal.pone.0087293

Denley, M. C. S., Gatford, N. J. F., Sellers, K. J., & Srivastava, D. P. (2018). Estradiol and the development of the cerebral cortex: An unexpected role? Frontiers in Neuroscience, 12, 245. https://doi.org/10.3389/fnins.2018.00245

Evsyukova, I., Plestant, C., & Anton, E. S. (2013). Integrative mechanisms of oriented neuronal migration in the developing brain. Annual Review of Cell and Developmental Biology, 29, 299–353. https://doi.org/10.1146/annurev-cellbio-101512-122400

Fenlon, L. R., Liu, S., Gobius, I., Kurniawan, N. D., Murphy, S., Moldrich, R. X., & Richards, L. J. (2015). Formation of functional areas in the cerebral cortex is disrupted in a mouse model of autism spectrum disorder. Neural Development, 10, 10. https://doi.org/10.1186/s13064-015-0033-y

Fester, L., Ribeiro-Gouveia, V., Prange-Kiel, J., vonSchassen, C., Bottner, M., Jarry, H., & Rune, G. M. (2006). Proliferation and apoptosis of hippocampal granule cells require local oestrogen synthesis. Journal of Neurochemistry, 97, 1136–1144. https://doi.org/10.1111/j.1471-4159.2006.03809.x

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 and Tissue Research, 280, 561–574. https://doi.org/10.1007/BF00318360

Gillies, G. E., & McArthur, S. (2010). Estrogen actions in the brain and the basis for differential action in men and women: A case for sex-specific medicines. Pharmacological Reviews, 62, 155–198. https://doi.org/10.1124/pr.109.002071

Hojo, Y., Hatori, T. A., Enami, T., Furukawa, A., Suzuki, K., Ishii, H., T., ... Kawato, S. (2004). Adult male rat hippocampus synthesizes estradiol from pregnenolone by cytochromes P45017alpha and P450 aromatase localized in neurons. Proceedings of the National Academy of Sciences of the United States of America, 101, 865–870.

Hojo, Y., & Kawato, S. (2018). Neurosteroids in adult hippocampus of male and female rodents: Biosynthesis and actions of sex steroids. Frontiers in Endocrinology, 9, 183. https://doi.org/10.3389/fendo.2018.00183

Jones, K. A., Sumiya, M., Woolfrey, K. M., Srivastava, D. P., & Penzes, P. (2019). Loss of EPAC2 alters dendritic spine morphology and the basis for differential action in men and women: A case for sex-specific medicines. Pharmacological Reviews, 62, 513–516. https://doi.org/10.1152/jn.00271.2018

Jossin, Y., & Cooper, J. A. (2011). Reelin, Rap1 and N-cadherin orient the migration of multipolar neurons in the developing neocortex. Nature Neuroscience, 14, 697–703. https://doi.org/10.1038/nn.2816

Kaczkurkin, A. N., Razmahan, A., & Satterthwaite, T. D. (2019). Sex differences in the developing brain: Insights from multimodal neuroimaging. Neuropsychopharmacology, 44, 71–85. https://doi.org/10.1038/s41386-018-0111-z

Kawachi, T. (2015). Cellular insights into cerebral cortical development: Focusing on the locomotion mode of neuronal migration. Frontiers in Cellular Neuroscience, 9, 394.

Konkle, A. T., & McCarthy, M. M. (2011). Developmental time course of estradiol, testosterone, and dihydrotestosterone levels in discrete regions of male and female rat brain. Endocrinology, 152, 223–235. https://doi.org/10.1210/en.2010-0607

Kretz, O., Fester, L., Wehrenberg, U., Zhou, L., Brauckmann, S., Zhao, S., ... Rune, G. M. (2004). Hippocampal synapses depend on hippocampal estrogen synthesis. Journal of Neuroscience, 24, 5913–5921. https://doi.org/10.1523/JNEUROSCI.5186-03.2004

Kubo, K., Tomita, K., Uto, A., Kuroda, K., Seshadri, S., Cohen, J., ... Nakajima, K. (2010). Migration defects by DISC1 knockdown in C57BL/6, 129X1/Sv1, and ICR strains via in utero gene transfer and virus-mediated RNAi. Biochemical and Biophysical Research Communications, 400, 631–637. https://doi.org/10.1016/j.bbrc.2010.08.117

Lenroot, R. K., Gogtay, N., Greenstein, D. K., Wells, E. M., Wallace, G. L., Clasen, L. S., ... Giedd, J. N. (2007). Sexual dimorphism of brain developmental trajectories during childhood and adolescence. NeuroImage, 36, 1065–1073. https://doi.org/10.1016/j.neuroimage.2007.03.053

Lu, Y., Sareddy, G. R., Wang, J., Wang, R., Li, Y., Dong, Y., ... Brann, D. W. (2019). Neuron-derived estrogen regulates synaptic plasticity and memory. Journal of Neuroscience, 39, 2792–2809. https://doi.org/10.1523/JNEUROSCI.1990.2019

MacLusky, N. J., Naftolin, F., & Goldman-Rakic, P. S. (1986). Estrogen formation and binding in the cerebral cortex of the developing rhesus monkey. Proceedings of the National Academy of Sciences of the United States of America, 83, 513–516. https://doi.org/10.1073/pnas.83.2.513

MacLusky, N. J., Walters, M. J., Clark, A. S., & Toran-Allerand, C. D. (1994). Aromatase in the cerebral cortex, hippocampus, and mid-brain: Ontogeny and developmental implications. Molecular and Cellular Neurosciences, 5, 691–698. https://doi.org/10.1006/mcne.1994.1083

Maeng, L. Y., Waddell, J., & Shors, T. J. (2010). The prefrontal cortex communicates with the amygdala to impair learning after acute stress in females but not in males. Journal of Neuroscience, 30, 16188–16196. https://doi.org/10.1523/JNEUROSCI.2265-10.2010

Martinez-Cerdeno, V., Noctor, S. C., & Kriegstein, A. R. (2006). Estradiol stimulates progenitor cell division in the ventricular and subventricular zones of the embryonic neocortex. European Journal of Neuroscience, 24, 3475–3488. https://doi.org/10.1111/j.1460-9568.2006.05239.x

McCarthy, M. M., Herold, K., & Stockman, S. L. (2018). Fast, furious and enduring: Sensitive versus critical periods in sexual differentiation of the brain. Physiology & Behavior, 187, 13–19. https://doi.org/10.1016/j.physbeh.2017.10.030

McCarthy, M. M., & Wright, C. L. (2017). Convergence of sex differences and the neuroimmune system in autism spectrum disorder. Biological Psychiatry, 81, 402–410. https://doi.org/10.1016/j.biopsych.2016.10.004

Meseke, M., Prols, F., Schmahl, C., Seебо, K., Kruse, C., Brandt, N., ... Rune, G. M. (2018). Reelin and aromatase cooperate in ovarian follicle development. Scientific Reports, 8, 8722. https://doi.org/10.1038/s41598-018-26928-x

Montelli, S., Peruffo, A., Zambenedetti, P., Rossipal, E., Giacomello, M., Zatta, P., & Cozzi, B. (2012). Expression of aromatase P450(AROM) in the human fetal and early postnatal cerebral cortex. Brain Research, 1475, 11–18. https://doi.org/10.1016/j.brainres.2012.08.010

Niwa, M., Kamiya, A., Murai, R., Kubo, K., Gruber, A. J., Tomita, K., ... Nabeshima, T. (2010). Knockdown of DISC1 by in utero gene transfer disturbs postnatal dopaminergic maturation in the frontal cortex and leads to adult behavioral deficits. Neuron, 65, 480–489. https://doi.org/10.1016/j.neuron.2010.01.019
Sellers, K. J., Denley, M. C. S., Saito, H., How to cite this article: Sellers KJ, Denley MCS, Saito A, et al. Brain-synthesized oestrogens regulate cortical migration in a sexually divergent manner. Eur J Neurosci. 2020;52:2646–2663. https://doi.org/10.1111/ejn.14755

Srivastava, D. P., Woolfrey, K. M., & Penzes, P. (2013). Insights into rapid modulation of neuroplasticity by brain estrogens. *Pharmacological Reviews*, 65, 1318–1350. https://doi.org/10.1124/pr.111.005272

Tuscher, J. J., Szinte, J. S., Starrett, J. R., Krenzelt, A. A., Fortress, A. M., Remage-Healey, L., & Frick, K. M. (2016). Inhibition of local estrogen synthesis in the hippocampus impairs hippocampal memory consolidation in ovariectomized female mice. *Hormones and Behavior*, 83, 60–67. https://doi.org/10.1016/j.yhbeh.2016.05.001

Varghese, M., Keshav, N., Jacot-Descombes, S., Warda, T., Wicinski, B., Dickstein, D. L., … Hof, P. R. (2017). Autism spectrum disorder: Neuropathology and animal models. *Acta Neuropathologica*, 134, 537–566. https://doi.org/10.1007/s00401-017-1736-4

Waddell, J., Bangasser, D. A., & Shors, T. J. (2008). The basolateral nucleus of the amygdala is necessary to induce the opposing effects of stressful experience on learning in males and females. *Journal of Neuroscience*, 28, 5290–5294. https://doi.org/10.1523/JNEUROSCI.1129-08.2008

Wang, L., Andersson, S., Warner, M., & Gustafsson, J. A. (2003). Estrogen receptor (ERbeta) knockout mice reveal a role for ERbeta in migration of cortical neurons in the developing brain. *Proceedings of the National Academy of Sciences of the United States of America*, 100, 703–708.

Yague, J. G., Munoz, A., deMonasterio-Schrader, P., Defelipe, J., Garcia-Segura, L. M., & Azcoitia, I. (2006). Aromatase expression in the human temporal cortex. *Neuroscience*, 138, 389–401. https://doi.org/10.1016/j.neuroscience.2005.11.054

Yague, J. G., Wang, A. C., Janssen, W. G., Hof, P. R., Garcia-Segura, L. M., Azcoitia, I., & Morrison, J. H. (2008). Aromatase distribution in the monkey temporal neocortex and hippocampus. *Brain Research*, 1209, 115–127. https://doi.org/10.1016/j.brainres.2008.02.061

Yan, L., Qi, W., Liu, Y., Zhou, F., Wang, Y., Bai, L., … Wang, Q. (2019). The protective effect of aromatase on NSC-34 cells with stably expressed hSOD1-G93A. *Neuroscience*, 411, 37–46. https://doi.org/10.1016/j.neuroscience.2019.05.022

Zhang, L., Hernandez, V. S., Swanin, J. D., Verma, A. K., Giesecke, T., Emery, A. C., … Eiden, L. E. (2018). A GABAergic cell type in the lateral habenula links hypothalamic homeostatic and midbrain motivation circuits with sex steroid signaling. *Translational Psychiatry*, 8, 50. https://doi.org/10.1038/s41398-018-0099-5

Srivastava, D. P., Woolfrey, K. M., Liu, F., Brandon N. J., Penzes P. (2010). Estrogen Receptor Activity Modulates Synaptic Signaling and Structure. *Journal of Neuroscience*, 30, (40), 13454–13460. http://dx.doi.org/10.1523/jneurosci.3264-10.2010

SUPPORTING INFORMATION

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