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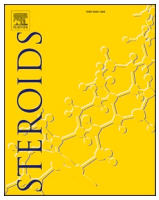

Estrogen and testosterone secretion from the mouse brain

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ABSTRACT

Estrogen and testosterone are typically thought of as gonadal or adrenal derived steroids that cross the blood brain barrier to signal via both rapid nongenomic and slower genomic signalling pathways. Estrogen and testosterone signalling has been shown to drive interlinked behaviours such as social behaviours and cognition by binding to their cognate receptors in hypothalamic and forebrain nuclei. So far, acute brain slices have been used to study short-term actions of 17β-estradiol, typically using electrophysiological measures. For example, these techniques have been used to investigate, nongenomic signalling by estrogen such as the estrogen modulation of long-term potentiation (LTP) in the hippocampus. Using a modified method that preserves the slice architecture, we show, for the first time, that acute coronal slices from the prefrontal cortex and from the hypothalamus maintained in aCSF over longer periods i.e. 24 h can be steroidogenic, increasing their secretion of testosterone and estrogen. We also show that the hypothalamic nuclei produce more estrogen and testosterone than the prefrontal cortex. Therefore, this extended acute slice system can be used to study the regulation of steroid production and secretion by discrete nuclei in the brain.

1. Introduction

Estrogen and androgens drive sexually dimorphic social behaviours and influence cognition via both genomic and non-genomic signalling mechanisms involving transcription and rapid signalling pathways respectively [1,2]. Estrogens act on estrogen receptors (ERs) in a conserved set of interconnected nuclei called the social behaviour network (SBN) which includes several areas of the hypothalamus, the bed nucleus of the stria terminalis (BNST) and the lateral septum [3,4]. These signalling mechanisms in the central nervous system (CNS) have been investigated in a variety of different model systems, including in the zebrafinch, quail [5] and in the rodent [6,7]. Typically, these studies have focused on the receptor and the actual signalling pathway involved in driving the behaviour [8,9], particularly in areas of the SBN, with the implicit idea that these pathways are regulated by gonadal steroids. However, in some model systems, notably in birds, the steroid hormones themselves are postulated to be brain-derived and may act in a synaptocrine manner [10] to drive behaviour.

Circulating levels of the predominant estrogen, 17β-estradiol, and the androgen, testosterone, vary between males and female vertebrates with estradiol generally being higher in females and testosterone being higher in males. However, in the brain, significant levels of testosterone and 17β-estradiol (17β-E), higher than the plasma, have been reported in several vertebrate species [11–13 5,14,15]. In the male songbird, where this has been extensively investigated, 17β-E measured by retrodialysis was increased in the auditory nidopallidum (NCM) upon acoustic stimuli i.e. birdsong and by social interaction with females [16]. Inhibition of aromatase activity in the NCM decreased 17β-E levels and led to the inability of the male zebrafinch to distinguish its own song from other songs [17]. Furthermore, administration of an aromatase inhibitor into the hippocampus decreased both acquisition and retention of spatial memories [18] in the zebrafinch. Likewise, in the male quail, administration of an aromatase inhibitor decreased appetitive sex behaviour [19]. In male and female mice, the forebrain-specific conditional deletion of the aromatase allele resulted in a reduction in spatial memory, recognition memory, spine and synapse density [20]. These studies in both birds and rodents demonstrate that local production of 17β-E in the brain has functional consequences, though the sexually dimorphic nature of the production and how production of these local steroid hormones is regulated is still not well understood.

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Acute and cultured, organotypic brain slices are widely used consolidated *ex vivo* models to investigate how hormones can regulate the function of single neurons and local neural networks in a native neural system, such as the mammalian brain [21]. Acute slices are obtained by quickly removing the brain from a euthanized animal, usually a mouse or a rat, at any age, placing it into ice cold cutting solution and using a vibration microtome to obtain 250 to 400 μm slices. The cut slices are then maintained in oxygenated artificial cerebrospinal fluid (aCSF). The thickness is a fundamental factor, as the slice needs to be thick enough to maintain some degree of integrity of the cytoarchitecture and connectivity within the neural networks, but thin enough to enable the exchange of nutrients, particularly oxygen and glucose, by simple diffusion. Following the cutting phase, the slices are left to recover at room temperature for at least 90 min, to allow the tissue to recover and the inflammation to settle to a more or less stable level. Acute slices are mostly used to measure the electrophysiological function of single neurons and local networks, using either multi-electrode arrays (MEAs) or glass micropipettes filled with a conductive solution and mounted on microelectrodes. Both single cell (patch-clamp and sharp electrode) and field potential electrophysiological recordings can be performed on acute slices [22–24]. Though a widely used model, acute slices are not kept alive for long, usually up to 24 h at most [25,26]. Similarly, organotypic slices, are acute slices, mostly from embryos or newly born pups, which are subsequently cultured on membranes and maintained in cell media in incubators [27,28]). The young age of the animal allows the experimenter to keep the brain tissue alive for a longer time, up to weeks and in some cases months, enabling to test for example the effect of long-lasting treatments on the neuronal structure and function *ex vivo*. However, the trade-off here is that since the brain is in an early phase of development at the time of the sacrifice, the physiological cytoarchitecture and connectivity is progressively lost in culture [29]. Acute slices have been widely used to study the rapid and/or sexually dimorphic actions of estrogen in the brain, notably the modulation of long term potentiation in the hippocampus which has led to the elucidation of intracellular signalling pathways that are modified by brief administration of 17β-E [30–32]. However, it has been more difficult to study outputs that are modulated by 17β-E over longer time scales such as those critically controlled by genomic or integrated signalling [6] and the requirement for locally produced 17β-E.

The aim of this study was twofold a) To optimise a system for longer-term incubation of "acute" slices in order to measure stable steroid analytes using adult and pubertal mice,and b) to further use a sensitive ELISA technique to measure estrogen and testosterone from a known area in the SBN i.e. the hypothalamus, containing mostly the ventromedial hypothalamus (VMH) and medial amygdala (meA) and a non-SBN area, the medial prefrontal cortex (mPFC). Apart from the hypothalamus which is a classical target brain area for estrogen action, the mPFC was chosen because it is key, along with the hippocampus, in the consolidation of memories [33] and both ERα and ERβmediate synaptic transmission in the mouse PFC [34], suggesting that this area that is important for cognition is an estrogen target.

2. Methods and materials

2.1. Animals/chemicals

Male mice C57BL/6J (from Charles River or Envigo Inc, UK) of two different ages - Week 3–5 (pubertal) or Week 7–10 (adult) were maintained on a 12:12 light and dark cycle with food and water ad libitum. Males were chosen so that there are no estrous cycle confounds on neurosteroid measurements and because males have already been shown to have higher levels of both 17β-E and testosterone in the hippocampus than females, at the time of sacrifice, by LC-MS [35,36]. Animals (n = 4–8/time point or for age of animal) were used to analyse neurosteroid levels secreted by brain slices, maintained *ex-vivo*. Procedures used in this study are performed by competent personnel trained in Schedule 1 procedures as per the Animal Scientific Procedures Act (1986, UK) at the University of Reading. All chemicals were obtained from Fisher Scientific, UK unless otherwise noted.

2.2. Dissection of the brain of the adult and pubertal male mice

Upon terminal isoflurane anesthesia and after cervical dislocation, the brain was dissected and quickly placed in vials containing 5 ml of ice-cold 30 % sucrose solution bubbled with 95 % O_2 and 5 % CO_2 (carbogen) for 2 min. The brain was then glued on the stage and placed into the cutting chamber of the vibratome (VT 1000S Leica, Wetzlar, Germany) containing carbogenated 30 % ice-cold sucrose solution. 200 μm-thick coronal slices of the medial prefrontal cortex (Bregma 2.22–1.54 mm; 4 slices of PFA per animal) and the hypothalamus (Bregma −1.22 to −2.06 mm; 4–5 slices per animal) were quickly cut using the vibratome, using the Paxinos Mouse Brain Atlas as guide [37]. Hence, the hypothalamic slices mainly encompassed the VMH and the meA while the medial prefrontal cortex (mPFC) encompassed the prelimbic and infralimbic nuclei.

In initial experiments done to optimise the procedure and identify the best balance between slice thickness, analyte concentrations that can be measured in the aCSF and viability, 300- μm sagittal or 150-μm coronal slices of the adult male hypothalamus were also taken. All slices, regardless of thickness, were gently removed using a Pasteur pipette and placed in sterile glass vials containing 5 ml of sterile artificial cerebrospinal fluid (aCSF; 12.9 mM NaCl, 300 μM KCl, 120 μM $MgSO_4$, 40 μM K_2HPO_4, 2.5 mM HEPES, 10 mM glucose, 1.4 mM $CaCal_2$, 0.4 mM ascorbic acid, pH 7.6) that had been oxygenated for 15 mins and placed at 37 °C for recovery for 45 min-1 h; this is the zero-hour time point. For experiments where the volume of the aCSF was the independent variable, brain slices were placed in varying volumes from 1 ml to 5 ml of oxygenated, warm, sterile aCSF in sterile vials and viability and hormones measured from the aCSF using a specific ELISA (Section 2.6), 24 h after incubation. For experiments where time-points were the independent variable, brain slices were gently transferred from the recovery vials and placed in 12 well plates containing 1.5 ml of sterile aCSF/well that had been preincubated at 37C for 30 mins. Each well with a slice represents a time point. The aCSF was then removed at the time points noted in the figures and stored at −20 °C prior to being assayed for 17β-E or testosterone. At the same time point, solid phase extraction (Section 2.5) was used to extract steroids either from the whole slice itself or from micropunches of specific brain areas from the slice (Section 2.4). In some initial experiments, slices were also used to measure viability (Section 2.3). Anterior to posterior positioned slices of the mPFC and hypothalamus from each animal were counterbalanced across different independent variables in each experiment.

2.3. Measurement of slice viability

aCSF was removed from the brain slices at different time points and the brain slices rinsed twice with phosphate buffered saline (PBS) at RT for 5 mins for each rinse. Slices were then placed in 0.4 % trypan blue for 4 min, after which slices were fixed with 4 % paraformaldehyde (PFA) for 30 min. Fixed brain slices were rinsed with PBS for 10 min twice at RT for 5 mins, placed on a clean glass slide, and coverslipped. The slide with the brain slice was viewed through the 10x objective lens on the Zeiss inverted microscope Axioscope (Carl Zeiss MicroImaging GmbH) and brightfield images of four areas of the prefrontal cortex and hypothalamus for a slice were taken under identical exposure times and gain settings. Stained cells were identified in 8-bit images and thresholded to remove background. Measurement of the percentage of the area occupied by these stained cells was carried out using the Measure function in Image J. Percentage viability was calculated as [1-(Stained area/Total area)] × 100, as per the protocol in [38].

2.4. Isolation of brain nuclei by Palkovits punches

For the male mouse mPFC which has not been investigated for neurosteroid production previously, we also wanted to further delineate the production of 17β-E and testosterone by the prelimbic and infralimbic nuclei. Hence, we used the Palkovits punch technique to specifically isolate these nuclei from multiple slices spanning these areas that were incubated at the 0- and 24-hour timepoints. This technique has been used previously for neurosteroid quantification from songbird brain [39]. After rinsing the incubated slices in 0.1 M PBS at RT, the slices were fixed using 4 % paraformaldehyde (PFA) for 20 min. The brain slices were rinsed with (PBS) 3 times and carefully placed on a calibration slide. This slide was then placed in a small container containing dry ice to partially freeze the slice to allow for firm micropunches to be made. Using a dissection microscope to identify the mPFC, a 0.5 mm punch tool (FineTools Inc, USA) was used to punch out the prelimbic (PL) and infralimbic (IL) area and carefully placed in an Eppendorf tube on dry ice and stored at −80 °C, until solid phase extraction (SPE) could be carried out. SPE (Section 2.5) was followed by ELISA (Section 2.6) for 17β-E and testosterone.

2.5. Solid phase extraction of steroids from tissue

This SPE method is optimised from the method used previously to extract neurosteroids from brain tissue with high recovery rates and reproducibility [40,41,39,42]. Either the entire coronal slice (200 μm) of the hypothalamus or mPFC or the PL and IL punches derived from the slice were homogenised in glass tubes with ice-cold 85 % HPLC-grade methanol (punches: 300 μl; slice: 1 ml) and left at 4 °C overnight. Non-end capped C18 columns (Stratech Inc, UK; for punches: 1 ml volume; for slice: 6 ml volume) were primed with absolute ethanol (1 ml column: 3 ml; for 6 ml column: 10 ml) and subsequently equilibrated twice with deionised water (for punches: 2 × 2.5 ml; for slice: 2 × 5 ml). Homogenised samples were diluted 1:10 in deionised water and loaded onto the equilibrated C18 column, taking care not to allow the column to run dry. After washing the column twice with deionised water (2 × 2.5 ml for punches, 2 × 5 ml for slice), steroids were eluted with 90 % HPLC-grade methanol (1.5 ml for punches/5 ml for slice). Steroid eluates were completely dried by a SpeedVac and the pellet was resuspended in 650 μl or 350 μl of 0.1 M phosphate-buffered saline containing 0.1 % (w/v) gelatin (PBSG) and 0.7 % (v/v) ethanol [42] (Fig. 1). Since elution volumes, number of micropunches/area and slice location and sizes were kept consistent, it is possible to compare across the treatment groups.

2.6. Enzyme linked immunosorbent assay (ELISA) assays for 17β-E and testosterone

This was carried out using sensitive in-house competitive ELISAs with antibodies against 17β-estradiol and testosterone. The 510/6 goat anti-oestradiol serum (kindly gifted by the late Prof G. S. Pope, National Institute for Research in Dairy, Shinfield, Berkshire, UK) was diluted 1/10,000 in 0.05 M carbonate buffer, pH 9.6. Likewise, the S505 mouse monoclonal anti-testosterone antibody (Clone MO21812, Fitzgerald Inc, USA), UK) was diluted 1/30,000 in 0.05 M carbonate buffer (pH 9.6). Neither antibody was stored at 4 °C for longer than 1 month prior to use.

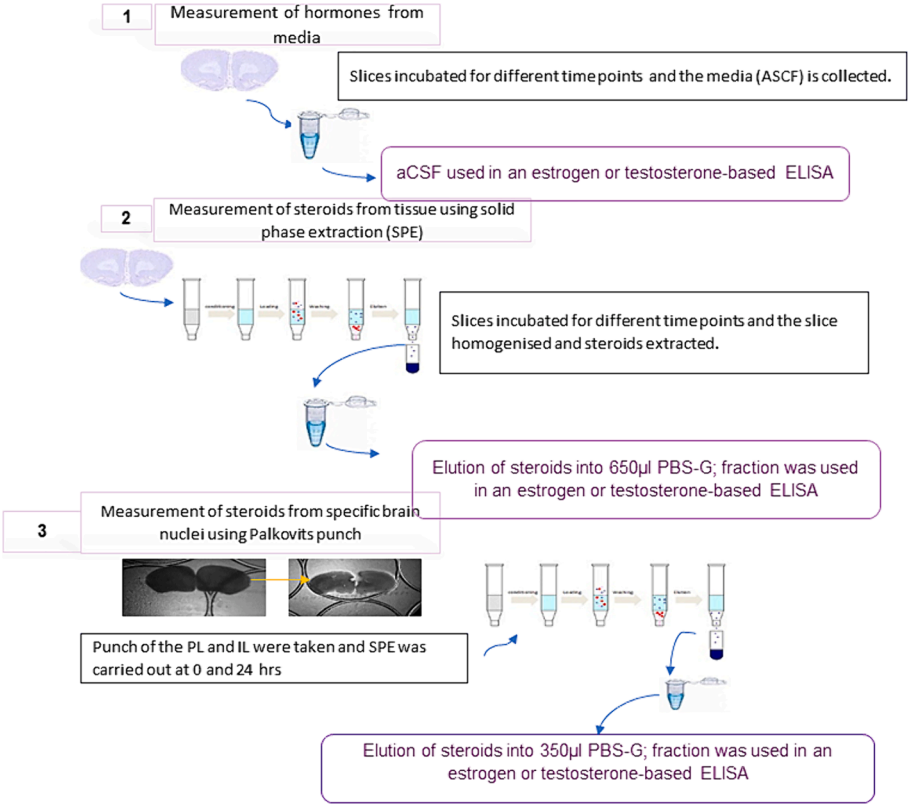

Fig. 1. Experimental setup of acute slices and subsequent workflow and analyses for steroid analytes. Steroids were measured in three different workflows, originating from three different sources 1) In most experiments, including those that used volume or time points as an independent variable, hormones secreted into the artificial cerebrospinal fluid (aCSF) were measured by a sensitive estrogen or testosterone-based competitive ELISA 2) Slices were used either for viability (initial experiments) or were extracted in the whole using solid phase extraction (SPE) and subsequent analyses using the estrogen or testosterone based competitive ELISA 3) In a single experiment with 200 μm coronal slices of the adult male prefrontal cortex, we generated micropunches using the Palkovits method of the infralimbic and prelimbic cortex across multiple slices. These were then extracted using SPE in the same manner as 2) and analysed using the estrogen or testosterone-based competitive ELISA.

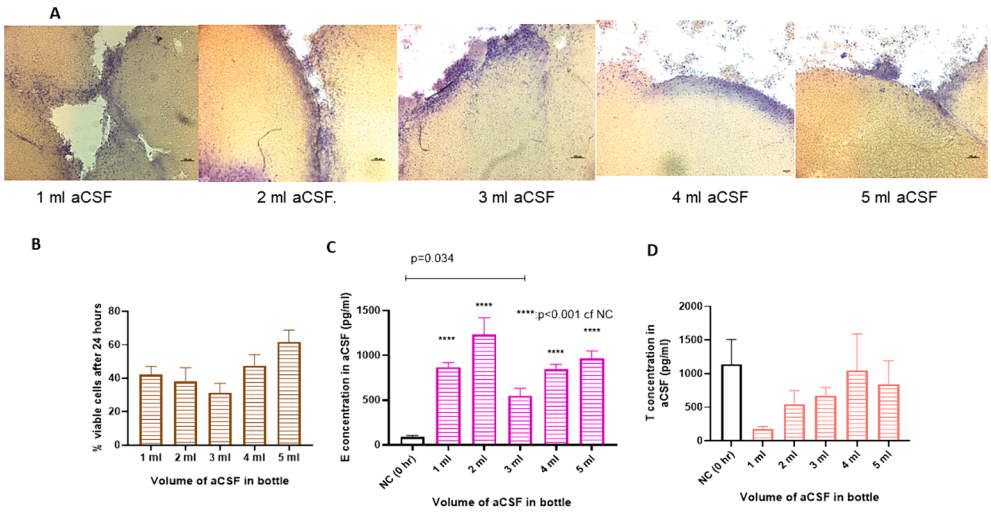

Fig. 2. Viability and steroidogenic capacity in sagittal slices of the hypothalamus of the pubertal male mouse. Mice were sacrificed and 300 μm hypothalamic slices corresponding to the VMH were incubated for 24 h in different volumes of oxygenated aCSF in sterile glass vials, as detailed in Methods. At the end of this incubation period, A) trypan blue staining was performed B) viability quantitated for n = 4 animals for each group and C) 17β-estradiol and D) testosterone measured from the aCSF bathing the slice. The negative controls for C) and D) are estradiol and testosterone levels measured from the 0-hour time point, as detailed in Methods and all volumes were compared to the negative control (NC); n = 4–7 animals/group. Since data distributions are normal, all data were analysed using one-way ANOVA followed by Sidak posthoc comparison test between groups. ****: $p < 0.0001$ cf negative control (NC). The statistic used to represent the data is represented as mean ± SEM. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Nunc MaxiSorp™ high protein-binding capacity 96 well ELISA plates (Fisher Inc, UK) were coated with 100 μl/well of the diluted anti-estradiol or anti-testosterone antibody and incubated overnight at room temperature in a moist, sealed plastic box. Excess antibody was washed off with 3 washes of PBS buffer containing 0.1 % (v/v) Tween-20 (PBS-T). Free binding sites in every well were blocked using 250 μl of PBS buffer containing 0.1 % gelatin and Proclin biocide (GPB) and the plates stored overnight in a moist, sealed box at 4 °C. Plates were used within a week of this procedure. On the day of use, plates were washed with 3 washes of PBS-T. Standards (made from a 1 μg/ml stock of testosterone or 17β-estradiol) ranged from 10000 pg/ml-0 pg/ml in 3-fold serial dilutions made in GPB. To prevent matrix effects, the volumes of samples are counterbalanced. 50 μl of standard (diluted in GPB) or 10 μl sample from each group were added to wells in duplicate and the volume made up to 60 μl by adding 50 μl GPB to sample wells and 10 μl aCSF to standard wells, thus ensuring an equivalent composition. The competing labelled antigens were E2-HRP (6-CMO-estradiol-HRP; catalog number: 65-1E16) or *T*-HRP (Testosterone-3-CMO-HRP; catalog number:65-IT07) (Fitzgerald Inc, USA). 50 μl of diluted estradiol (1:2000) or testosterone hapten (1:6000) in GPB, was added as the competing antigen to all wells except the blank wells. A set of duplicate blank wells i.e. 100 μl GBP + 10 μl "blank media" with the blank media being GBP (the diluent of the standards) or aCSF (the sample diluent) was also added to the plate. The plate was then mixed on a plate shaker and incubated for 3–4 hoursat room temperature in a moist, sealed box.

After incubation, the plate was washed again 3 times with PBS-T and 200 μl/well of freshly prepared HRP substrate i.e., o-phenylenediamine (OPD), diluted 1 mg/ml in 0.05 M citrate–phosphate buffer, pH 5.0, with 1 μl/ml 30 % hydrogen peroxide. The plates were incubated at room temperature in the dark for 30 min to allow the yellow colour to develop. 50 μl/well of 1 M hydrochloric acid was added to stop the reaction. The optical density or absorbance of each well was read at 490 nm (600 nm reference filter) on a microplate reader (Emax Microplate Reader, Molecular Devices, USA). Calibration curve fitting and calculation of 'unknowns' was carried out using the PC-based Softmax program (Molecular Devices) linked to the plate reader. Sample concentrations were adjusted for the appropriate dilution factor, prior to data analyses (Section 2.9 below). The intra and interassay variation for these assays is less than 9 % and < 12 % respectively for 17β-E and < 10 % and < 15 % respectively for testosterone. The assay detection limit was ~ 10 pg/ml for 17β-estradiol and ~ 15 pg/ml for testosterone. The specificity of the 17β-E assay was confirmed by determining the percentage cross-reactivity relative to the estradiol standard (100 %) of a range of steroids including pregnenolone (0.69 %), progesterone (0.06 %), dehydroepiandrosterone (0.13 %), testosterone (0.10 %), androstenedione (<0.05 %), dihydrotestosterone (0.21 %), estrone (7.5 %), deoxycorticosterone (<0.18 %), cortisol (<0.05 %), corticosterone (<0.05 %) and aldosterone (0.11 %). The specificity of the testosterone assay was confirmed by determining the percentage cross-reactivity relative to the testosterone standard (100 %) of a range of steroids including pregnenolone (0.02 %), progesterone (<0.01 %), dehydroepiandrosterone (0.05 %), androstenedione (1.32 %), dihydrotestosterone (6.25 %), estradiol-17β(0.61 %), estrone (0.04 %), deoxycorticosterone (<0.18 %), cortisol (<0.01 %), corticosterone (<0.01 %) and aldosterone (<0.01 %). A workflow for this process is in Fig. 1 and standard curves for both steroids are provided as Supplementary Fig. 1. In addition, since slices are incubated in aCSF, we also show that standards diluted in aCSF do not show any difference from standards in GPB for either 17β-E or testosterone (**Supplementary** Fig. 2**A and B**). Two samples i.e. aCSF from male animals (named B24.2 and C1) also showed parallel curves when tested in the assay at three-fold dilutions (3 serial dilutions done for each sample) (**Supplementary** Fig. 2**C and D**).

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.steroids.2024.109398.

2.7. Relative quantitation of gene expression using real time PCR

RNA was isolated from micropunches of the IL and PL from each animal after storage in 5 volumes of RNA*later* Stabilization Solution (Invitrogen, Fisher Scientific) at −20C. Samples were processed within 3 weeks of collection for optimal RNA recovery. Total RNA was extracted using a Qiagen RNeasy Plus Mini Kit, following manufacturer's instructions. Absorbance at A_{260} and A_{280} nm was measured using the NanoDrop™ Spectrophotometer (Thermo Fisher Scientific) to determine RNA concentration and purity. 100 ng total RNA was reverse transcribed in a 20 μl reaction mixture using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Thermo Fisher

Scientific), following manufacturer's instructions. A single reaction mixture contained 2 μl reverse transcription buffer, 2 μl random primers, 0.8 μl deoxyNTP mixture, and 1 μl MultiScribe reverse transcriptase. The StepOne™ Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific) was used to create the following reaction conditions: annealing at 25 °C for 10 min, reverse transcription at 37 °C for 120 min, RNA denaturation at 85 °C for 5 min.

2.8. qPCR

qPCR was performed in MicroAmp™ Fast Optical 96-well Reaction Plates (Applied Biosystems, Thermo Fisher Scientific) with the StepOne™ Real-Time PCR System. The cDNA was amplified in a reaction volume of 14 μl, consisting of 7 μl 2X *Power* SYBR® Green Master Mix (Thermo Fisher Scientific) and primers at a final concentration of 360 nM. cDNA was added at 5 ng concentration/well and the reaction volume made up to 14 μl with nuclease-free water. The primer sets were designed by Integrated DNA Technologies Primer 3 tool and checked against NCBI Primer-Blast for duplex formation with other species or other genes. Primer sequences for all target genes are as follows: StAR Forward: 5′ GGAACCCAAATGTCAAGGAGATCAAGG 3′ StAR Reverse: 5′ AGGCCCCACCAGGTTGCC 3′ and Cyp19a1 forward: 5′ GGACACCTCTAACATGCTCTTCCTGGG 3′ and Cyp19a1 reverse: 5′ TGATGAGGAGAGCTTGCCAGGC 3′. For each cDNA sample, PCR amplification of theβ-actin housekeeping gene was determined to allow normalisation between the samples. Both target genes were amplified in duplicate by the same conditions: an initial step of 95 °C for 10 min, 40 cycles of denaturation at 94 °C for 15 s, followed by annealing and extension at 60 °C for one minute. The Ct values for aromatase and StAR mRNA from the IL and PL are compared to that of obtained from adult male hippocampal samples using relative quantification, with the $2^{-\Delta\Delta CT}$ method [43]. We used hippocampus as a positive control since aromatase expression is high in this area in males [44] and it is functionally relevant for spatial memory [45].

2.9. Data analyses

For all hormones measured directly from the aCSF or for hormones extracted by SPE from all brain slices or from punches obtained from the mPFC, technical duplicates from each sample were averaged and the mean obtained from all samples in the treatment group. For hormones measured from 10 μl aCSF at different volumes (Fig. 2), hormone concentration was normalised for each different volume to account for the dilutional effect at higher volumes and reported as pg/ml. For experiments where micropunches of the PL and IL from the mPFC were used, steroid concentrations were expressed as pg/volume of tissue (in mm^3) using the following formula for cylindrical three-dimensional punches of 200-μm depth:

$$V(mm3) = \pi x 0.5^2 x(0.2\ x\ the\ number\ of\ slices\ the\ nucleus\ spans)$$

For punches that encompassed the infralimbic and prelimbic areas, we took 8 punches (4 punches per nuclei per hemisphere) from across 4 slices for each nuclei/animal. All data were plotted in Graph Pad Prism (v8; Graph Pad Prism Inc, USA) as column graphs. Normality testing on data and lognormal data was carried out using the Shapiro Wilk test; distributions that are not normal are plotted as median ± min and max while normal data is plotted as mean ± SEM. For analyses of incubation volume, all volumes were compared to the 0-hour time point for 17β-E and testosterone analyses, using one-way ANOVA followed by Sidak's posthoc comparison test since these data were normally distributed. For time point analysis of steroids secreted by the hypothalamic coronal slices, non-parametric data was analysed using Kruskal-Wallis followed

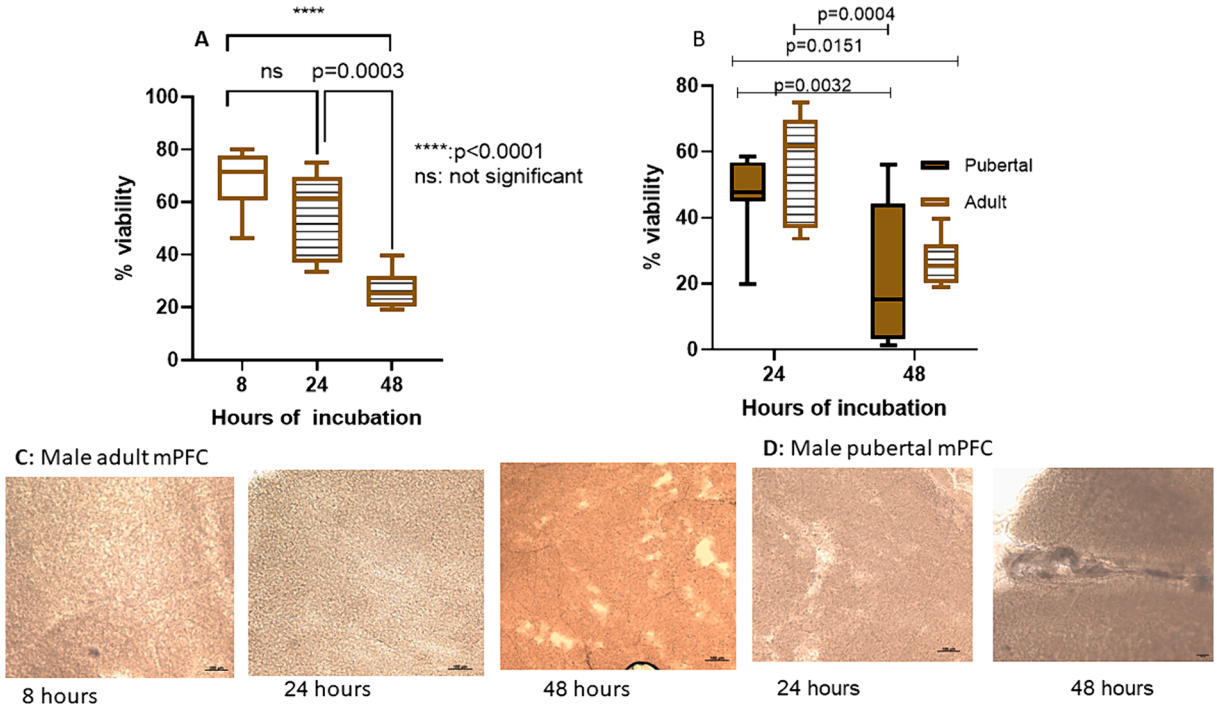

Fig. 3. Viability of 200 μm coronal slices obtained from the male mouse prefrontal cortex from the adult and pubertal stages. Pubertal and adult mice were sacrificed and 200 μm coronal slices obtained and incubated in sterile aCSF for varying periods of time, as detailed in Methods. A) Viability data, as assessed by trypan blue staining, in adult mPFC at different time points (n = 8 animals/group). This data is normally distributed and hence this was analysed using a one-way ANOVA followed by Sidak's posthoc comparison test. B) Comparison of viability between pubertal and adult male PFC 200 μm coronal slices (n = 8 mice/group). These data were analysed using a two-way ANOVA which revealed no significant effect of developmental stage (puberty vs adult) but revealed a significant effect of the hours of slice incubation ($p < 0.0001$). Posthoc comparisons were done using Tukey's multiple comparison test. C-D) Images of adult (Panel C) and pubertal (Panel D) male mPFC slices taken using 10x objective (Zeiss Axioskop). Data are represented as median ± min/max. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

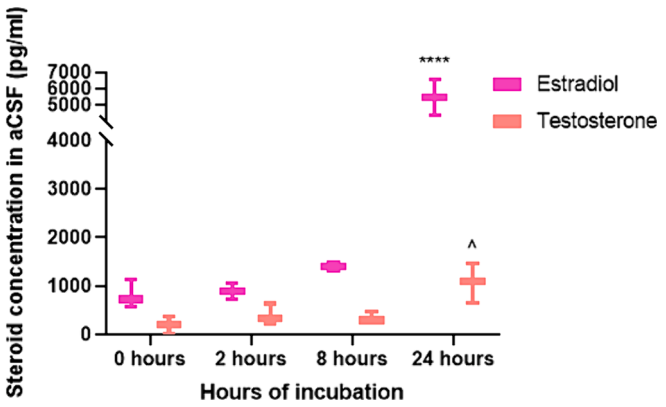

Fig. 4. 17β-estradiol and testosterone measured in aCSF bathing the adult male hypothalamic slice at different time points. Adult male mice were sacrificed and 200 μm coronal slices obtained and incubated in sterile aCSF for varying periods of time, as detailed in Methods, for the hypothalamus. Hypothalamic slices mainly encompassed the VMH and meA; (n = 4 mice/group). Data were analysed using a two-way ANOVA followed by Tukey's multiple comparison test, and both the time of slice incubation as well as the steroid (E vs T) was significant ($p < 0.0001$). In addition, the interaction between these two factors was also significant ($p = 0.0002$). There was a tendency towards an increase in testosterone concentrations at 24 h when compared to 0 h (^: $p = 0.09$ cf 0 h control) while 17β-E concentrations at 24 h were higher than testosterone or estrogen concentrations at every time point (****:$p < 0.0001$ cf all time points, irrespective of steroid). Data is represented as median ± min/max.

by the Dunn's posthoc comparison test. For comparisons of viability between pubertal and adult slices or between adult mPFC and adult hypothalamus between 0- and 24-hour time points, two-way ANOVA followed by the Tukey's posthoc comparison test for time and developmental stage was used for analyses. For comparisons of estrogen and testosterone concentrations between time points of male adult hypothalamus or mPFC or between pubertal mPFC and adult mPFC, two-way ANOVA followed by either Tukey's or Sidak's multiple posthoc comparison test for developmental stage or brain region and steroid was used for analyses. A $p < 0.05$ was considered significant.

3. Results

3.1. Both sagittal and coronal slices of the adult and pubertal mouse brain are capable of steroidogenesis over 24 h

In the first set of experiments (Fig. 2), we used 300-μm sagittal slices from the pubertal mouse hypothalamus (Fig. 2**A**) to investigate both steroidogenesis and viability. Fig. 2**B** shows that slices were equally viable when incubated in varying volumes of sterile aCSF in glass vials for 24 h; there was no significant difference between groups. All slices showed steroidogenic activity – i.e. slices showed increased secretion of 17β-E (Fig. 2**C**) but not testosterone (Fig. 2**D**) at 24 h when compared to the zero-hour time point, independent of aCSF volume. Next, we wanted to investigate steroid concentrations in the mouse mPFC, an area that has not been investigated for local steroidogenesis previously. However, aromatase in this region has been shown to increase in the human Alzheimer brain [46]. Since we also wanted to use coronal, rather than sagittal, slices to investigate steroid concentrations in different micropunches, we first investigated if viability was maintained over 48 h in the adult and pubertal male mouse mPFC. Viability declined from 24 to 48 h in both adult (Fig. 3**A and C**; $p = 0.0003$ for 24 h timepoints compared to the 48-hour timepoint) and pubertal male slices (Fig. 3**B and D**; $p = 0.0032$ for viability between 24 and 48 h). Furthermore, 200 μm coronal brain slices from the adult male hypothalamus tended to show increased steroidogenic capacity i.e., greater testosterone only at 24 h compared to 0 h (Fig. 4; $p = 0.09$ at 24 h compared to 0 h) with 17β-E concentrations being secreted at 24 h are at higher levels than testosterone and 17β-E at all time points (Fig. 4; $p < 0.0001$). Hence, in subsequent experiments using adult or pubertal male brain slices, we

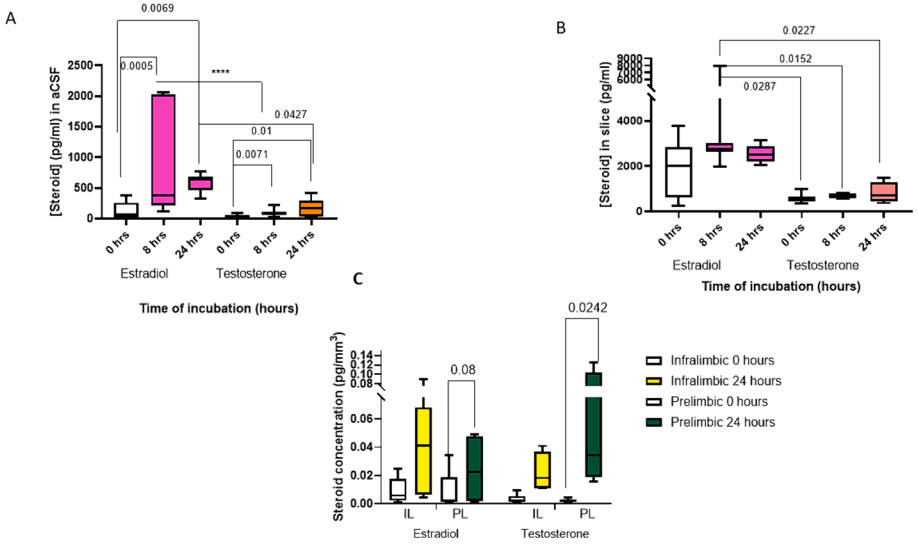

Fig. 5. 17β-estradiol and testosterone levels in the adult male mPFC slice at different time points. Adult male mice were sacrificed and 200 μm coronal slices obtained and incubated in sterile aCSF for varying periods of time, as detailed in Methods, for the medial prefrontal cortex. A) In the aCSF bathing the adult mPFC, there was increased estrogen and testosterone with increasing time; (n = 8 mice/group). Data were analysed using a two-way ANOVA followed by Tukey's multiple comparison test, which revealed a significant effect of both time of slice incubation and steroid (E vs T) ($p < 0.0001$). There was also a significant effect of interaction between these two factors ($p = 0.0010$). Testosterone concentrations were lower than estrogen concentrations. B) This increase from 0 h was not seen at 24 h for either testosterone or estrogen concentrations obtained after SPE of the whole slice; (n = 8 animals/group). Data were analysed using a two-way ANOVA followed by Tukey's multiple comparison test, which revealed a sole significant effect of steroid (E vs T) ($p = 0.0004$). C) The adult male mPFC slice was further dissected using the Palkovits punch technique and steroid hormone concentrations estimated in the punches after being subject to SPE; (n = 5–8 animals/group). Data were analysed using a two-way ANOVA followed by Tukey's multiple comparison test, which revealed a significant effect of time of incubation of the slice ($p = 0.0003$).As can be seen, testosterone increased only in the prelimbic nucleus at 24 h while 17β-E levels did not change in the infralimbic or prelimbic nuclei within 24 h, though there was a trend to significance for estradiol increase in the prelimbic nuclei ($p = 0.08$) between 0 and 24 h. Data is represented as median ± min/max.

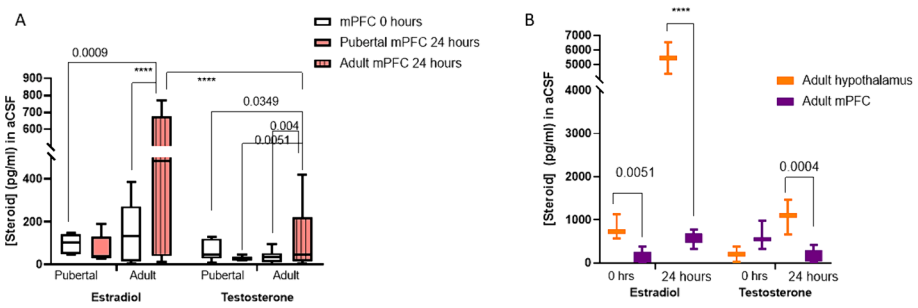

Fig. 6. Comparison of estrogen and testosterone concentrations obtained from aCSF bathing male brain slices at different developmental stage or between different areas of the adult male brain. Adult or pubertal male mice were sacrificed, and 200-μm coronal slices obtained and incubated in sterile aCSF for 24 h, as detailed in Methods. Estrogen and testosterone were measured in the aCSF at 0- and 24-hour timepoints. A) Comparison between adult and pubertal male mice (n = 6 animals/group). Data were analysed by two-way ANOVA followed by Tukey's multiple comparison test with the interaction between steroid × developmental stage ($p = 0.0244$) and the interaction between developmental stage × hours of incubation significant ($p = 0.0032$). Estrogen was higher at 24 h when compared to 0-hour time points in the adult mPFC but not in the pubertal mPFC. Estrogen at the 24-hour time point was also higher than testosterone at any developmental stage or timepoint. B) Comparison of steroid concentration between the adult mPFC and the adult hypothalamus at different time points (n = 6 animals/group). Data were analysed by two-way ANOVA followed by Tukey's multiple comparison test with the interaction between steroid × area of the brain, steroid × time of incubation ($p < 0.0001$) and the interaction between area of the brain × time of incubation also significant ($p < 0.0001$). Estrogen concentration at 24 h was higher than any other estrogen or testosterone concentration at 0 or 24 h, irrespective of brain region (****: $p < 0.0001$). Testosterone concentration at 24 h in the hypothalamic slice was significantly higher than testosterone concentration at 0 h in the same slice ($p = 0.0228$) and also higher than testosterone levels at 0 or 24 h in the mPFC ($p < 0.0001$). Data are represented as median ± min and max. ****: $p < 0.0001$ in both panels.

probed secreted 17β-estradiol and testosterone levels predominantly in the aCSF at 0- and 24-hour time-points.

In adult male mPFC slices, 17β-E and testosterone increased from 0 to 24 h in the aCSF (Fig. 5**A**) but did not increase when extracted from the entire slice itself using SPE methods (Fig. 5**B**). Again, like the scenario in the adult male hypothalamic slice (Fig. 4C), levels of 17β-E were higher than testosterone at 24 h (Fig. 5**B**). The 24-hour increase in testosterone was most likely due to the increase of testosterone in the prelimbic cortical nuclei (Fig. 5**C**) at 24 h rather than in the infralimbic nuclei.

3.2. The adult hypothalamus is more steroidogenic than the adult mPFC

We also compared the 17β-E and testosterone levels secreted into the aCSF by the adult and pubertal brain. Both hormones increased only in the adult mPFC at 24 h (Fig. 6**A**; levels of 17β-E at 24 h were significantly different from all other groups), with 17β-E levels greater than testosterone at 24 h in the adult mPFC (Fig. 6**A**). Testosterone levels at 24 h in the adult mPFC were also higher than testosterone levels at 0 h in the adult or pubertal mPFC and higher than the concentration seen at 24-hour levels in the pubertal mPFC. Furthermore, the adult hypothalamus is more steroidogenic than the adult mPFC since both 17β-E and testosterone showed higher levels in the aCSF at 24 h than their counterpart mPFC slices (Fig. 6**B**; $p < 0.0001$ for 17β-E and testosterone at 24 h in the adult hypothalamus compared to the mPFC at 0 or 24 h or the hypothalamus at 0 h).

4. Discussion

4.1. Methodology of acute slice preparation and steroid assay

As far as we are aware, our protocol is the first to show steroidogenic activity of mouse hypothalamic and medial prefrontal cortex coronal slices beyond the traditional acute-slice timeframe of 6–8 h. Our initial experiments, based on a modification of Loryan *et al*, used sagittal 300-μm slices from the pubertal hypothalamus in different volumes of highly oxygenated aCSF in a bottle for 24 h. As per the original protocol, we were careful to maintain the pH, high oxygenation and ice-cold conditions while processing the tissue [47], irrespective of the thickness of the slice. Our results also show that 17β-E measured from the aCSF at 24 h was significantly increased in all the incubation volumes, suggesting that slices are viable and steroidogenically active. This increase was specific to 17β-E since testosterone did not increase in the same time frame in the aCSF.

Though our initial study was done using hypothalamic sagittal 300-μm slices, we further optimised the technique to use coronal slices of 200 μM, to aid recovery of steroids from different brain nuclei. In male mPFC from either adult or pubertal mice, viability was roughly similar at 24 h but declined sharply at 48 h. Similar rates of viability were also found in 300-μm rat parasagittal slices where decreased temperature of incubation of the slice and irradiated aCSF decreased bacterial load [48] and prolonged viability. Like our study, Hupp *et al* [49] did not use antibiotics but did not see any bacteria if aseptic aCSF (as in our study) was used. In addition, this study and ours used mouse coronal sections, similar recovery times with carbogenation after slicing on the vibratome and incubation in 12 well plates in a tissue-culture type incubator [49]. Thickness of the slice was also important since most studies, including ours, use > 200-μm; our attempt at using 150-μm coronal slices yielded non-functional slices after 24 h (**Supplementary** Fig. 3).

Our assay for 17β-E and testosterone is a sensitive in-house competitive ELISA assay which is easy to conduct for everyday use, though liquid chromatography tandem mass spectrophotometry (LC-MS) and GC–MS is the assay of choice if sample volumes are small or precious, or if multiple steroids are to be measured. For example, LC-MS was used to assay the higher levels of progesterone, dehydroepiandrosterone, 5α-DHT, testosterone and pregnenolone in circulating plasma of domesticated versus wild zebra-finches. However, a sensitive radioimmunoassay was sufficient to detect higher levels of testosterone in the plasma of wild females and male zebra finches versus captured animals [50]. Our solid phase extraction (SPE) method is a minor modification of the method detailed by Newman *et al* who demonstrated that SPE could extract steroids such as dehydroepiandrosterone and 17β-E efficiently from the lipid-rich brain tissue of the songbird as well as from plasma with removal of interfering substances [39]. A uniform SPE treatment to samples prior to ELISA assay can be used to compare across these samples. This has been used for the time-dependent measurement of steroid hormones in bovine follicles [51] and for yolk-estrogen levels across egg laying in the female zebra finch [52]. Organic solvent use in the SPE, prior to the ELISA, also improved accuracy of detection of testosterone when compared to a reference LC-MS method in mouse testis extracts and in serum [53].

4.2. Hypothalamic and cortical slices from adult male mice secrete estrogen and testosterone

Our results show that both 17β-E and testosterone levels increased in the aCSF after 24 h of incubation of the adult male coronal hypothalamic (mainly VMH/meA) and the mPFC slices. However, this finding is subtly different from the levels of steroid hormones extracted from the adult male mPFC slice by SPE. Neither 17β-E nor testosterone showed statistical increases at the 24-hour timepoint, which could be attributed to the high basal level of these steroids at 0 h from the slice (**Supplementary** Fig. 4**A and 4B**). Though steroids may not be stored in tissue, they could be in the process of active synthesis in the tissue and hence the tissue concentrations for both 17β-E and testosterone are much higher than the steroid secreted in the aCSF. Supporting this, testosterone, measured by LC-MS was far lower in the CSF than in the male rat cerebral cortex, cerebellum or hippocampus while 17β-E was lower in the CSF than in the cerebellum, suggesting that levels in the brain tissue are generally higher than those found in the CSF bathing the brain [54]. In addition, many steroids in the CSF, though correlative, could not adequately predict levels in the tissue, though the hypothalamus and prefrontal cortex were not specifically investigated [54,55] as in this study.

In the hypothalamic slice from male adult mice, the 17β-E (**Supplementary** Fig. 4**C**) but not testosterone (**Supplementary** Fig. 4**D**) increase in the aCSF over 24 h was paralleled by the increase seen in the extracted steroid from the slice. These differences between mPFC and hypothalamic slices could also be seen in the far higher capacity of the hypothalamus to synthesize and secrete both 17β-E and testosterone. This could be due to the higher aromatase expression in the male meA that is part of this slice [44] while aromatase fibres, from the AH, meA, LS and BNST also synapse onto the VMH [56,57] and could release 17β-E into the VMH that is also present in this hypothalamic slice. In the male quail, testosterone induction of aromatase activity was maximal in the VMH compared to other aromatase-positive nuclei [58]. Indeed, in the songbird brain, though 17β-estradiol concentrations at baseline were similar between males and females, they were much higher in the NCM that in other areas of the pallium [59], suggesting that 17β-E varies to a greater extent within different regions within a sex rather than sex-specific differences. The relative level of steroidogenic enzymes in the mouse adult male mPFC is not known and is being currently studied in our laboratory, as is the dependence of this increase on aromatase inhibition. Hence, we have generally compared steroid levels from the aCSF between different time points, brain regions or age (Figs. 4-6).

4.3. Secreted levels of testosterone versus 17β-estradiol in the adult male mPFC and the hypothalamus

Most studies of steroid levels in the rodent brain have measured steroids from tissue of gonadally intact or gonadectomised/adrenalectomised animals, after sacrifice, in combination with localisation of steroidogenic enzymes to suggest that the steroid is synthesized *de-novo* from cholesterol [12,60]. This has been studied to the largest extent in the hippocampus [13]. For example, in the hippocampus, a sensitive derivatisation technique for steroids prior to LC-MS measurement in gonadally intact rats demonstrated that hippocampal 17β-E, testosterone and DHT levels were higher in the adult rat hippocampus than in the serum, with testosterone levels double that of 17β-E in the hippocampus [61]. In male adult rats, testosterone levels measured in cerebral cortex, hippocampus, cerebellum, and the spinal cord were higher than 17β-E while in female rats, 17β-E was marginally higher than testosterone [54].

In our study, both adult male mouse hypothalamus and mPFC slices showed higher levels of 17β-E than testosterone in males. Since other studies, including those mentioned above, measured steroids in rat tissue immediately after sacrifice, this difference could reflect species differences, differential steroidogenic capacity due to *ex vivo* slice incubation and/or differential regulation of steroidogenic capacity in different areas of the brain, as compared to the hippocampus and cerebral cortex. Indeed, though the neurons of the hippocampus express 17α-hydroxylase and aromatase, as detected by RT-PCR and immunohistochemistry, these enzymes are expressed to a greater extent in the hypothalamus than the hippocampus [12] and may account for higher conversion of testosterone to 17β-E in the hypothalamus [11]. Supporting this contention, in the adult male rat hypothalamus, there were far higher levels of aromatase mRNA, as detected by RT-PCR [62] or protein as detected by immunohistochemistry of a aromatase-promoter-GFP transgenic mouse, when compared to the hippocampus while the cerebral cortex expressed lower levels of aromatase mRNA [62] and protein [44] compared to the hippocampus Additionally, in some mouse strains such as the C57BL/6 used here, there are relatively low levels of circulating androgens [63 64] which may influence the testosterone levels found in the slice, at least at 0 h. We also show that mRNA levels of aromatase (*Cyp19a1*) and of the steroid acute regulatory protein (*StARD1*) exist at low levels in the prelimbic and infralimbic nuclei, compared to the hippocampus (**Supplementary** Fig. 5). In songbirds, male songbirds make 17β-E in the CNS that diffuses into the plasma and guarantees that serum levels of 17β-E are equivalent between males and females [65]. In a single similar study where, secreted steroids were measured from the aCSF that was bathing 400 μm thick adult male rat hippocampal slices, 2 h after slicing on the vibratome, secreted 17β-E and DHT were about 0.5 nM compared to about the 5–10 nM measured immediately from the hippocampus after sacrifice. Testosterone measured in the same manner was 1 nM compared to about 18 nM after sacrifice [61]. This suggests that secreted steroids decrease in the short term after mechanical stress. Our data, at least for the adult male hypothalamus sagittal slice also showed no increase at the 2-hour time point (**Supplementary** Fig. 3); this could be due to the slice being in a "recovery" period after the mechanical slicing procedure [61].

It is also possible that testosterone, but not 17β-E, levels are mainly controlled by gonadal steroids. In the cerebral cortex, long-term and short-term castration reduced both progesterone and testosterone levels in male and female adult rats [66]; 17β-E was not measured in this study. Furthermore, though testosterone and DHT in the adult male rat hippocampus, measured on sacrifice, decreased on castration, 17β-E did not, demonstrating that these two steroids are differentially regulated by gonadal status [67]. In incubated slices that are free of gonadal influences, this may mean a conversion of testosterone to 17β-E by aromatase with no replenishment of testosterone. Furthermore, the levels of 17β-E in the hippocampus were double that of testosterone in castrated rats and these levels are in a similar range as the concentration of 17β-E in the adult male hypothalamic slices, in our study.

4.4. 17β-estradiol and testosterone levels in the pubertal mice mPFC

As far as we are aware, no study has investigated local estrogen production in the rodent brain during the pubertal period after weaning. Data from the mPFC slices from pubertal male mice were different from those obtained from adult male mice, despite similar viability. Though 17β-E and testosterone levels, measured from the aCSF, were not significantly different at 0 h between the pubertal and adult mPFC, both were significantly higher at 24 h in the aCSF obtained from the adult mPFC compared to the aCSF bathing the pubertal mPFC slice. This is likely because 17β-E and testosterone did not increase in pubertal slices during the 24-hour incubation as compared to the adult. Though steroidogenesis in the pubertal mouse brain is poorly understood, a single study has investigated the developmental progression of 17β-E and testosterone concentration in the male and female rat brain. 17β-E and testosterone levels were higher in the embryonic brain compared to the postnatal brain with the cortex and hypothalamus higher than the hippocampus. After birth, levels in all areas steadily declined, with no difference readily apparent between pubertal and young adult mice [68]. However, in these studies, samples were processed immediately after sacrifice to measure steroids; it is possible that steroidogenic

capacity in *ex-vivo* slices is different. For example, 5α-reductase expression was higher in puberty than in the adult [69] giving rise to the possibility that testosterone is converted to reduced catabolites, leading to low levels of both testosterone and 17β-E during this developmental period. In addition, aromatase mRNA levels detected by qPCR in one area of the hypothalamus, the bed nucleus of the stria terminalis (BNST), were relatively constant from postnatal day 20–60 [70] though the levels in the pubertal mPFC remain unknown. Apart from testosterone conversion to estradiol and 5-dihydrotestosterone, both estradiol and testosterone can be hydroxylated by the *Cyp1A* enzymes, mainly expressed in the liver [71]. However, the *Cyp1A1* isoform is expressed in the brain, albeit at a low level [72]; the relative expression of this in adult or pubertal prefrontal cortex and hypothalamus are unknown. To test this possibility, comparison of the relative level of all steroidogenic and catabolic *Cyp* enzymes in pubertal versus adult mPFC slices as well as measurement of reduced catabolites, and steroidogenic intermediates from aCSF using LC-MS will need to be done.

5. Conclusion

Our method not only allows for the measurement of analytes secreted by any region of the mouse brain, but also for in-depth analyses of analytes in specific nuclei using micropunches and subsequent extraction. A more involved method i.e., dispersion cultures of rat hippocampal cells cultured in serum-free media also demonstrated that17β-E that was secreted into the media increased over time [73], while letrozole reduced production. In rodents, an organotypic method has been described for newborn rats where 400-μm hippocampal slices have been incubated for a week in media supplemented with horse serum. Addition of letrozole to this culture decreased the number of synapses and the number of presynaptic boutons [74], suggesting that estrogens can be made locally in slice-type preparations and are important for the maintenance of synaptic density. The function of local neuroestrogens, possibly via *denovo* synthesis in the male mPFC is currently a focus of study in our laboratory.

Our method using tissue from adult mice would also allow for the simultaneous measurement of analytes in the aCSF along with other correlates – for e.g., Golgi staining for neuromorphology, or immunohistochemistry or enzyme assays for target proteins in the slice. Furthermore, one could study integrated signalling i.e. the interplay of both rapid nongenomic signalling and slower genomic signalling in a more physiological context than dissociated cell cultures. Using this method, we uniquely report the increase in secreted 17β-E and testosterone production over time in the male adult mouse prefrontal cortex. In addition, we propose that the younger brain may produce lower concentrations of select neurosteroids compared to the adult brain.

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CRediT authorship contribution statement

Ruby Vajaria: Methodology, Formal analysis. **DeAsia Davis:** Methodology, Formal analysis, Data curation. **Kongkidakorn Thaweepanyaporn:** Methodology, Formal analysis, Data curation. **Janine Dovey:** Writing – original draft, Methodology, Data curation. **Slawomir Nasuto:** Supervision. **Evangelos Delivopoulos:** Funding acquisition. **Francesco Tamagnini:** Writing – original draft, Methodology. **Philip Knight:** Writing – review & editing, Supervision, Methodology. **Nandini Vasudevan:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Project administration, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.steroids.2024.109398.

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