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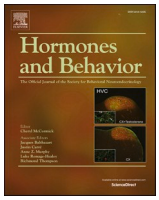

Comparative analysis of gonadal hormone receptor expression in the postnatal house mouse, meadow vole, and prairie vole brain

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A B S T R A C T

The socially monogamous prairie vole (*Microtus ochrogaster*) and promiscuous meadow vole (*Microtus pennsylvanicus*) are closely related, but only prairie voles display long-lasting pair bonds, biparental care, and selective aggression towards unfamiliar individuals after pair bonding. These social behaviors in mammals are largely mediated by steroid hormone signaling in the social behavior network (SBN) of the brain. Hormone receptors are reproducible markers of sex differences that can provide more information than anatomy alone and can even be at odds with anatomical dimorphisms. We reasoned that behaviors associated with social monogamy in prairie voles may emerge in part from unique expression patterns of steroid hormone receptors in this species, and that these expression patterns would be more similar across males and females in prairie than in meadow voles or the laboratory mouse. To obtain insight into steroid hormone signaling in the developing prairie vole brain, we assessed expression of estrogen receptor alpha (*Esr1*), estrogen receptor beta (*Esr2*), and androgen receptor (*Ar*) within the SBN, using in situ hybridization at postnatal day 14 in mice, meadow, and prairie voles. We found species-specific patterns of hormone receptor expression in the hippocampus and ventromedial hypothalamus, as well as species differences in the sex bias of these markers in the principal nucleus of the bed nucleus of the stria terminalis. These findings suggest the observed differences in gonadal hormone receptor expression may underlie species differences in the display of social behaviors.

1. Introduction

Mammalian species show a wide diversity of social behaviors. This natural variation can be leveraged to identify the unique circuitry that imparts a behavior of interest. Several vole species are established model organisms for the study of sociality (Carter et al., 1995; He et al., 2019; Insel and Young, 2001; Lee and Beery, 2019; Sadino and Donaldson, 2018). There are stark behavioral differences between monogamous and promiscuous vole species: prairie voles display social monogamy, biparental care, and form long-lasting pair bonds, which rarely occur in mammals, while promiscuous meadow voles are socially non-monogamous, uniparental, and seasonally social in same-sex groups (Anacker et al., 2016; Beery, 2019). Notably, social monogamy is accompanied by decreased behavioral sex differences in prairie voles compared to mice and rats. Male prairie voles care for offspring and juveniles and show high levels of spontaneous alloparenting (Bales et al., 2006; Carter and Getz, 1993; Getz et al., 1981; Kramer et al., 2009; Lonstein and De Vries, 2000). Prairie voles also display similar levels of aggression in females and males. Males attack novel conspecifics at low levels, but upon pair-bond formation, both sexes show selective aggression towards strangers (Getz et al., 1981; Lee and Beery, 2022; Tickerhoof et al., 2020; Wang et al., 1997; Young et al., 2011). Thus, a pair-bond encompasses both prosocial (towards the partner) and anti-social (towards novel conspecifics) behaviors.

Social behaviors, such as aggression, mounting, lordosis, and parenting behaviors, are mediated by the social behavior network (SBN), which processes pheromonal cues to determine social context and select the appropriate response (Chen and Hong, 2018; Goodson, 2005; Newman, 1999; Wallace et al., 2023). In many species, including humans, several regions of the SBN show sexual dimorphism in cell density, cell number, volume, projection pattern, or gene expression, particularly the sexually dimorphic nucleus of the preoptic area (SDN-POA), the principal nucleus of the bed nucleus of the stria terminalis (BNSTpr), and anteroventral periventricular nucleus (AVPV) (Allen and Gorski, 1990; Gorski et al., 1978; Kelly et al., 2013; Simerly et al., 1985; Tsukahara and Morishita, 2020). In mice and rats, these dimorphisms are organized by perinatal testosterone signaling in males. Circulating testosterone is locally converted to 17β-estradiol in select neuronal populations that express aromatase and this neural estradiol drives brain sexual differentiation (Balthazart and Ball, 1998; Juntti et al., 2010;

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Lephart, 1996; MacLusky and Naftolin, 1981; Naftolin and Ryan, 1975; Wu et al., 2009). Intriguingly, monogamous species such as prairie voles also show decreased sexual dimorphism in the brain, as well as in anatomical measures such as body weight and anogenital distance (Campi et al., 2013; Dewsbury et al., 1980; Heske and Ostfeld, 1990; Shapiro et al., 1991). In addition, prairie vole social behaviors are resistant to early life testosterone manipulations, as if the classic organization and activation model is operating under different constraints in this species. Like other rodents, prairie vole males undergo a perinatal testosterone surge (Lansing et al., 2013), but in contrast to rats, neonatal orchiectomy does not abolish adult male sexual behavior. Instead, postnatal testosterone *reduces* androgen induced mounting behavior in adult males (Roberts et al., 1997). Perinatal testosterone also does not masculinize the expression of arginine vasopressin (*Avp*) in the BNST and medial amygdala (MeA) (Lonstein et al., 2002). These findings suggest that prairie vole brain sexual differentiation follows a distinct trajectory compared to those of mice and rats (Bonthuis et al., 2010).

We reasoned that behaviors associated with social monogamy may emerge in part from unique expression patterns of gonadal steroid hormone receptors in prairie voles. Previous studies have investigated the distribution of ERα immunoreactivity across group-housed vole species in adulthood, demonstrating differences in overall ERα levels within the SBN, as well as decreased expression in males of socially-monogamous species, particularly in the MeA (Cushing et al., 2004; Cushing and Wynne-Edwards, 2006; Hnatczuk et al., 1994). However, male mice and rats also show lower expression of ERα/*Esr1* in males compared to females (Cao and Patisaul, 2013; Gegenhuber et al., 2022; Kanaya et al., 2018; Kelly et al., 2013; Xu et al., 2012; Yokosuka et al., 1997), and no studies have directly compared hormone receptor expression between voles and mice. In addition, although immunoreactivity to ERβ has been reported to be less sexually dimorphic than ERα in prairie voles (Ploskonka et al., 2016), AR expression in this species has only previously been described in males (Cushing et al., 2004).

To obtain insight into species differences in brain sexual differentiation, we carried out a systematic comparison of *Esr1*, *Esr2,* and *Ar*, using in situ hybridization (ISH) at postnatal day 14 (P14) in mice, prairie, and meadow voles. The inclusion of meadow voles permits comparisons within two *Microtus* species and provides a behavioral intermediate between polygamous mice and monogamous prairie voles: meadow voles show a partner preference for same-sex peers and engage in seasonal social group living (Anacker et al., 2016; Beery, 2019; Beery et al., 2014; Lee and Beery, 2022). We selected ISH to allow a consistent methodology across all three genes. Immunostaining for ERβ has been historically complicated by antibody variability; a previous comprehensive characterization of ERβ expression in mice utilized a transgenic *Esr2*-GFP line (Andersson et al., 2017; Nelson et al., 2017; Zuloaga et al., 2014), while experiments in prairie voles utilized an antibody raised against a prairie vole epitope (Ploskonka et al., 2016). We chose P14 as this time point is beyond the closure of the postnatal sensitive period for brain sexual differentiation in mice and rats (MacLusky and Naftolin, 1981; McCarthy, 2008; Simerly, 2002), precedes pubertal gonadal hormone secretion (Brock et al., 2011; Piekarski et al., 2017). P14 also coincides with a dynamic increase in the expression of the oxytocin receptor (Oxtr), a gene that underlies species differences in social behavior (Hammock and Levitt, 2013; Hammock, 2014; Insel et al., 1993; Johnson and Young, 2015; Newmaster et al., 2020). Since prairie voles in particular are more precocial in their early postnatal development than promiscuous species (Shapiro and Insel, 1990) in terms of tooth eruption, fur growth, and eye opening, we anticipated that the closure of the sensitive period in voles would be at an earlier postnatal age as in mice (or at the same time, but not a later age). We additionally investigated sex differences in hormone receptor expression in the postnatal BNSTp.

2. Methods

2.1. Animals

Prairie voles (*Microtus ochrogaster*) and meadow voles (*Microtus pennsylvanicus*) were bred in-house from lineages obtained from the labs of Devanand Manoli (at least 10 generations from wild-caught, UCSF) and Annaliese Beery (3 generations from wild-caught, Smith College/UC Berkeley), respectively. All voles were maintained on a 12:12 light cycle with lights on at 7 am, with bedding, nesting material (nestlet), and a PVC hiding tube, and provided food and water ad libitum. C57BL/6 J mice were chosen as a standard laboratory model organism for comparison and bred in-house, maintained on a 12:12 light cycle with lights on at 3 am, and provided food and water ad libitum, bedding, and nesting material. All animal procedures were performed in accordance with the Cold Spring Harbor Laboratory animal care committee's regulations.

Vole pups of both species were removed from their parental home cages and transcardially perfused with 4 % paraformaldehyde (PFA) on postnatal day 14 (P14) and tails were collected for genotyping by PCR for presence or absence of the *Sry* gene. C57BL6 mouse pups were perfused at P14 and categorized by anogenital distance. Brains were extracted, cryoprotected in 30 % sucrose, and stored at -80 °C until cryosectioning for histology.

2.2. Histology

Frozen brains were coronally cryosectioned at 50 μm, collected in RNase-free PBS, and mounted on VWR Superfrost Plus slides. In situ hybridization (ISH) using digoxigenin labeled RNA probes against androgen receptor (*Ar*), estrogen receptor alpha (*Esr1*), or estrogen receptor beta (*Esr2*) was performed as previously described (Gegenhuber et al., 2022) (details of the probes used for these genes can be found in Supplemental Table 1). Sections were post-fixed on slides with cold 4 % PFA for 20 min, rinsed, and treated with proteinase K (10 μg/mL, Roche) for 20 min at room temperature. Sections were then postfixed again for 5 min before treatment with acetylation buffer for 10 min and permeabilization in 1 % Triton for 10 min. Slides were then rinsed and sections equilibrated in hybridization solution for 2–4 h at room temperature. Sections were subsequently incubated for 16–20 h at 65 °C in fresh hybridization buffer containing 350 ng/mL probe and washed in 0.2× SSC buffer. Washes were followed by blocking in 10 % heat-inactivated sheep serum for 1–3 h and incubation in buffer containing sheep anti-digoxigenin antibody (Roche) at 1:5000 dilution for 16–20 h at 4 °C. After 2 h of repeated washing, slides were incubated at 37 °C in a staining solution containing nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate (Roche) for 20–24 h. Staining was stopped with 1 mM EDTA, and slides were washed, postfixed, and coverslipped.

2.3. Microscopy

All slides were imaged under brightfield illumination at 2× and/or 10× magnification and stitched using a Keyence BZ-X800 All-in-One Fluorescence microscope and Keyence BZ-X Viewer/Analyzer software. All 50 μm sections were imaged from anterior (Bregma 0.38 mm) to posterior (Bregma −2.80 mm). Processed images were analyzed by an investigator blinded to the sex of the animal and mean intensity of staining was quantified and normalized. In brief, a uniform mask was applied to all individuals for 5 bilateral sections (See Supplementary Figs. 2, 4, and 6) for each image in Adobe Photoshop 2022, utilizing the lateral and 3rd ventricles, anterior commissure, and fornix as landmarks. Using Fiji analysis software (NIH), each image was colour inverted, the background staining of each image was subtracted from the ROI, and the average pixel brightness of staining was quantified. These scores were then averaged bilaterally and summed for a total region intensity.

2.4. Statistical analysis

Statistical analysis was performed in Excel and all plots were made using the ggplot2 (v3.4.1) package in R v4.2.1 (https://cran.r-project.org/bin/windows/base/old/4.2.1/) (Wickham, 2011). Because intensity scores within a replicate were correlated with one another, we expressed the intensities as a ratio of male to female intensity scores for each replicate. Ratios greater than one indicate a male bias of the gene of interest, ratios less than one indicate a female bias, and ratios of one indicate no sex bias. We then performed a one-sample *t*-test within each species on the log of the ratios to determine if the log ratio was significantly different from 0.

2.5. Single nucleus RNA sequencing analysis

snRNA-seq data containing 1,228,636 single-cell 10×v2 transcriptomes across several brain regions and sex and corresponding metadata were accessed from The Allen Institute for Brain Science Brain Map Portal: https://portal.brain-map.org/atlases-and-data/rnaseq/mouse-whole-cortex-and-hippocampus-10x.

To analyze expression levels of hormone receptors (*Ar, Esr1, Esr2, Pgr,* and G-protein coupled estrogen receptor 1, *Gper1*), single-cell transcriptomes were subsampled by neighborhoods and subclasses according to the cell-type taxonomy established in Yao et al., 2021. Neighborhoods ("") and clusters (n_{start}-n_{end}) for each subsample are as follows: 1) Hippocampus, glutamatergic: "DG/SUB/CA" 318–364; 2) Cortex, GABAergic: "CGE, MGE" 5–123; 3) Cortex, layer 2/3 glutamatergic: "L2/3 IT" 124–177; 4) Cortex, layer 4/5/6 glutamatergic: "L4/5/6 IT Car3" 178–238; 5) Cortex PT glutamatergic: "PT" 239–263; 6) Cortex layer 6 glutamatergic: "NP/CT/L6b" 264–317. Cluster labels and abbreviations used in this analysis are provided in Supplemental Table 2. Subsample gene expression counts and metadata were read into R via HDF5 (rhdf5: (Fischer et al., 2023); HDF5Array: (Pagès, 2023)) and then loaded into a Seurat object (Satija et al., 2015). Counts were LogNormalized and scaled within each subsample. Subsamples were visualized with VlnPlot() by cluster labels, then exported and designed with Adobe Illustrator.

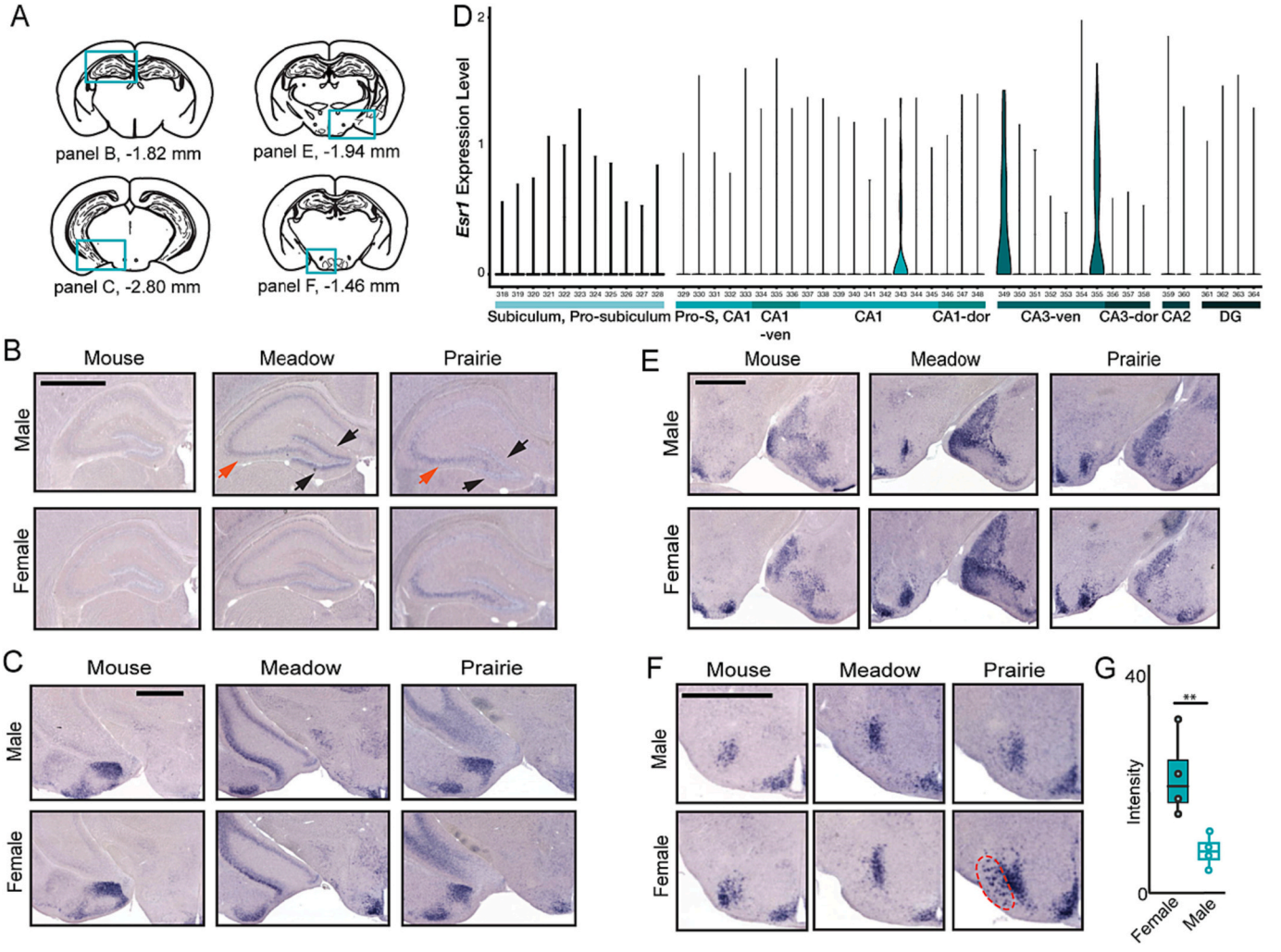

Fig. 1. Species differences in *Esr1* expression. (A) Schematics of mouse coronal sections for dorsal hippocampus (dHPC), ventral hippocampus (vHPC), medial amygdala (MeA), and ventromedial hypothalamus (VMH) (Paxinos and Franklin, 2019). (B—C) In situ hybridization (ISH) for *Esr1* expression in dHPC (B) and vHPC (C). Red arrowheads denote CA3, black arrowheads denote dentate gyrus. Note species differences in vHPC. Male (top panels) and female (bottom panels) are shown for mouse, meadow vole, and prairie vole (left to right). (D) Violin plot of *Esr1* expression across hippocampal cell types in adult B6 mice, organized by anatomical subregion. See Supplementary Table 2 for subclass abbreviations and cluster identity. (*E*-F) ISH for *Esr1* expression in MeA (E) and VMH (F). Red dotted lines outline VMHvll. (G) Quantification of VMHvll staining intensity (sum of 5 bilateral consecutive section averages) in prairie vole females and males. Boxplot denotes median and 1st & 3rd quartiles, ** $p < 0.01$. Scale bars = 1000 μm.

3. Results

3.1. Species and sex differences in Esr1 expression

Although overall localization of *Esr1* transcripts within the SBN was largely similar across species, several differences were readily apparent in the regions schematized in Fig. 1A. We first observed minimal *Esr1* staining within the mouse hippocampus (Fig. 1B,C). To corroborate this low signal, we turned to a large snRNA-seq dataset generated by the Allen Institute that encompasses the entire mouse isocortex and hippocampal formation of adult C57BL/6 J mice of both sexes (Yao et al., 2021). Across 47 clusters of hippocampal glutamatergic neurons, expression of *Esr1* appears in only three clusters of hippocampal excitatory neurons: (343_CA1, 349_Mossy, and 355_CA3_ventral), with relatively low expression even within these clusters (Fig. 1D). However, *Esr1* is also found within multiple GABAergic cell types, several of which are enriched in hippocampus compared to cortex: Lamp5-Lhx6+ clusters 5, 7, and 8; Sncg/Ntng1+ cluster 29; Vip + cluster 55; and Sst + clusters 102 and 104) (Yao et al., 2021) (Supplementary Fig. 1C).

In contrast, *Esr1* signal was strong in the hippocampus of both meadow and prairie voles, with striking enrichment in distinct subregions. In the dorsal hippocampus of meadow, but not prairie voles, *Esr1* is particularly abundant in the dentate gyrus (black arrowheads), yet is strongly expressed within CA3 only in prairie voles (red arrowheads) (Fig. 1B). In the ventral hippocampus, there is staining in the amygdalo-hippocampal and amygdalo-piriform area in both meadow and prairie vole (Fig. 1C). The full range of staining intensity is shown on full sections in Supplementary Fig. 1A. We noted an anatomical difference in that the ventral portion of the hippocampus extends dorsally from the ventral surface moving through posterior coronal sections (Supplementary Fig. 1B) in meadow voles. Cortical staining of *Esr1* was minimal in all three species and is not shown by ISH; this minimal staining is corroborated by the sparse expression of *Esr1* in both GABAergic and glutamatergic populations in adulthood (Supplementary Fig. 1C—H).

Esr1 is expressed in the medial amygdala (MeA), ventromedial hypothalamus ventrolateral region (VMHvl), and arcuate nucleus as shown in Fig. 1E. We found an unexpected sex difference in the localization of *Esr1* transcripts in prairie voles. In addition to the intense VMHvl staining typically seen in mice, females also express *Esr1* in a more lateral population (Fig. 1F, red dashed line). Quantification of *Esr1* expression in this VMHvll region showed a significant sex difference in a log ratio test (95 % CI (−0.724, −0.200); $p = 0.0014$; $N = 4$ animals per sex, Fig. 1G, Supplementary Fig. 2A). This region was quantified only in prairie voles because the anatomic VMHvll subdivision was not sufficiently distinguishable from the principal VMHvl expression in mice or meadow voles, suggesting a novel, prairie vole-specific sexual dimorphism.

Within the SBN of mice and rats, *Esr1* levels are higher in females compared to males, particularly within the VMHvl, MeA, POA, and BNSTpr (Gegenhuber et al., 2022; Kelly et al., 2013; Xu et al., 2012; Yokosuka et al., 1997). While assessing species differences in *Esr1* expression, we noted that sex differences appeared more subtle in both species of voles compared to mice. We selected the BNSTpr for quantitation as sex differences in this region have not previously been investigated in voles (Campi et al., 2013; Shapiro et al., 1991). As expected, we observed a robust sex difference in *Esr1* levels in the BNSTpr of mice (95 % CI (−0.203,-0.068); $p = 0.00054$, $N = 5$ animals per sex) (Fig. 2). However, we did not detect a significant difference between sexes in either meadow (95 % CI (−0.039,0.088); $p = 0.32$; N = 5 animals per sex) or prairie voles (95 % CI (−0.359,0.224); $p = 0.49$; N = 4 animals per sex). The log ratio of male to female expression was not significantly different from zero in either vole species (Fig. 2E). The quantified region (5 consecutive sections of principal nucleus only) is outlined in Supplementary Fig. 2B.

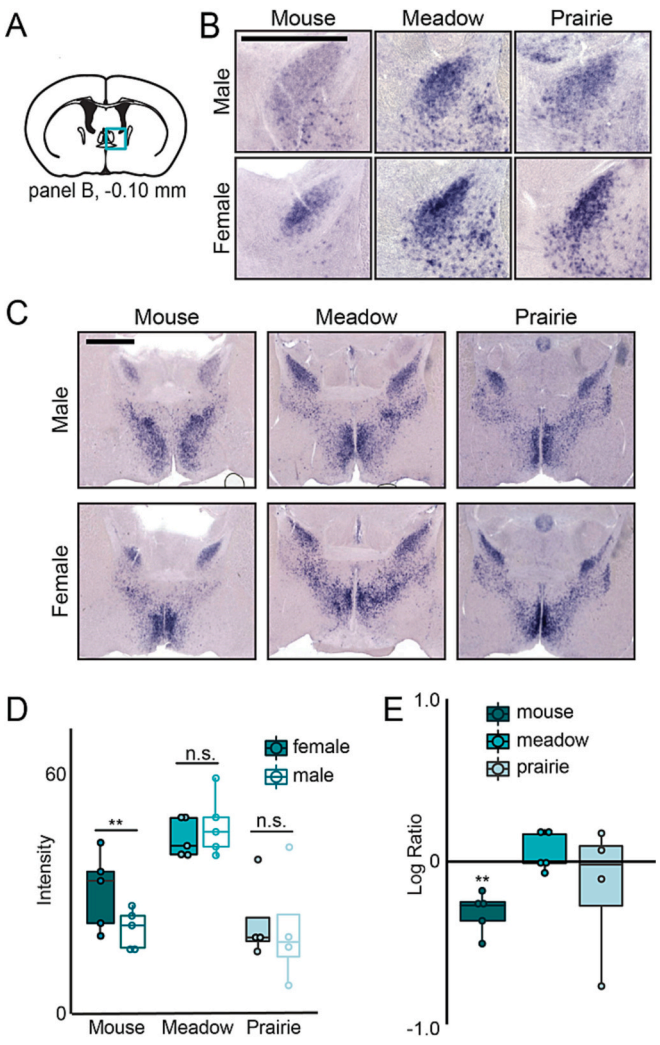

Fig. 2. Sex differences in *Esr1* expression. (A) Schematic of coronal section for bed nucleus of stria terminalis principal nucleus (BNSTpr) *Esr1* expression and quantification. (B) ISH for *Esr1* in BNSTpr. Male (top panels) and female (bottom panels) are shown for mouse, meadow vole, and prairie vole (left to right). (C) ISH for *Esr1* in BNST and preoptic area (POA) of the hypothalamus. (D) Quantification of BNSTpr *Esr1* expression (sum of 5 bilateral consecutive section averages) in males and females of each species ($N = 5$, mouse, meadow vole; $N = 4$, prairie vole). Boxplot denotes median and 1st & 3rd quartiles, ** $p < 0.01$. (E) Within-batch male:female log ratios of *Esr1* expression in BNSTpr. ** denotes significant difference from log ratio of zero. Scale bars = 1000 μm.

3.2. Species and sex differences in Ar expression

In mice and rats, *Ar* expression is more widespread than that of *Esr1* (Brock et al., 2015; Simerly et al., 1990). We performed ISH for *Ar* in our three species and identified differences within the regions schematized in Fig. 3A. As with *Esr1*, we found pronounced species differences in hippocampal *Ar* expression (Fig. 3B). While *Ar* is strongly expressed in the CA1 of all three species, high expression within CA2 is only seen in mice (gray arrows). In the CA3 (red arrows) and dentate gyrus (black arrows), *Ar* expression mirrors that of *Esr1*, with more expression in prairie voles compared to other species in CA3 and in dentate gyrus of meadow voles, as well as mice, compared to prairie voles. The strong expression of *Ar* within the mouse hippocampus is also seen by snRNA-seq, where *Ar* is present in most glutamatergic cell types (Fig. 3C).

Generally, *Ar* appears more diffusely expressed in prairie voles than meadow voles or mice, particularly in the MeA (Fig. 3D). The full range of staining intensity can be seen in Supplementary Fig. 3A. However, the

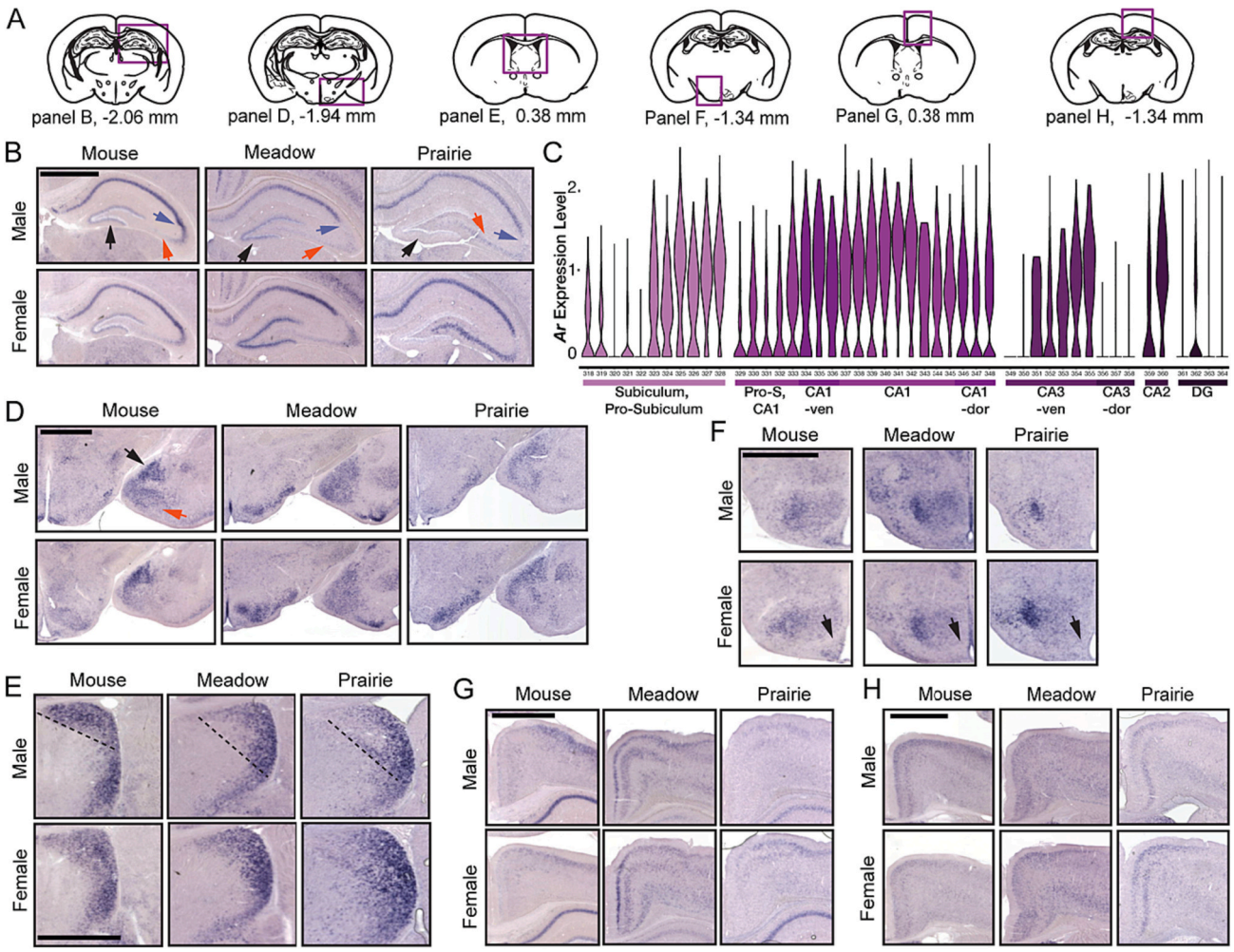

Fig. 3. Species differences in *Ar* expression. (A) Schematics of coronal sections for dorsal hippocampus (dHPC), medial amygdala (MeA), lateral septum (LS), ventromedial hypothalamus (VMH), anterior cingulate cortex (ACC), and retrosplenial area. (B) ISH for *Ar* in dHPC. Black arrows denote dentate gyrus, red arrows denote CA3, and blue-gray arrows denote CA2. Male (top panels) and female (bottom panels) are shown for mouse, meadow vole, and prairie vole (left to right). (C) Violin plot of *Ar* expression across hippocampal cell types in adult B6 mice, organized by anatomical subregion. See Supplementary Table 2 for subclass abbreviations and cluster identity. (D—H) ISH for *Ar* in MeA (D), LS (E), VMH (F), ACC (G), and retrosplenial area (H). Black arrow indicates MeApd, red arrow indicates MeApv. Dashed line indicates the dorsal LS region of interest. Black arrowheads indicate arcuate nucleus. Scale bars = 1000 μm.

density of subregions within the MeA is more defined in mice than in either vole species, with a clear distinction between the posterodorsal nucleus (MeApd, black arrow) and posteroventral nucleus (MeApv, red arrow). The distribution of *Ar* expression in the lateral septum (LS) is different between mouse and vole species, with *Ar* expression taking up a broader area of dorsal LS in voles than in mice (Fig. 3E). In mice, *Ar* expression is restricted to a thinner region of the dorsal lateral septum, while in voles the *Ar* expression in the dorsal region protrudes more medially and ventrally.

In the ventromedial hypothalamus, *Ar* is expressed throughout the VMH (including dorsomedial and central VMH) in the mouse (Fig. 3F, left). In the prairie vole, expression is more restricted to the VMHvl (Fig. 3F, right). Meadow voles have an intermediate expression pattern with broad expression throughout the VMH and enrichment of expression in VMHvl (Fig. 3F, center). Additionally, although *Ar* is expressed in the arcuate nucleus in mice, it is largely absent from meadow and prairie voles (Fig. 3F, black arrowheads).

Ar is much more prevalent throughout the cortex compared to *Esr1* (Supplementary Fig. 3B—D), both in the anterior cingulate cortex (Fig. 3G), as well as the more posterior retrosplenial area (Fig. 3H), Accordingly, in snRNA-seq data, *Ar* is present within multiple inhibitory neuronal types, barring parvalbumin interneurons, and is also prevalent in glutamatergic populations, particularly the deep layers (Supplementary Fig. 3C—H).

Both mice and meadow voles had a pronounced difference in *Ar* expression in the BNSTpr with male expression higher than females (mouse 95 % CI (−0.003,0.237); $p = 0.027$, meadow 95 % CI (0.033,0.183); $p = 0.0041$), while prairie voles had no significant differences in BNSTpr *Ar* expression (95 % CI (−0.225,0.231); $p = 0.97$), (Fig. 4, $N = 5$ animals per sex for all species). The region of BNSTpr quantification is outlined in 5 consecutive BNSTpr sections in Supplementary Fig. 4.

3.3. Species and sex differences in Esr2 expression

Esr2 expression in all three species is extremely sparse within the span of sections that we assessed (Fig. 5A) as well as by snRNAseq in the mouse hippocampus (Fig. 5B). As expected, *Esr2* is highly expressed in the paraventricular nucleus of the hypothalamus (PVN) of all three species (Fig. 5C). Notably, PVN expression in meadow voles extends through nearly twice as many coronal sections as in mouse and prairie vole, indicating a larger anterior-posterior distribution of *Esr2* expression in meadow vole PVN (not shown). *Esr2* is expressed in MeApd in mice, and more broadly throughout the posterior MeA in both vole

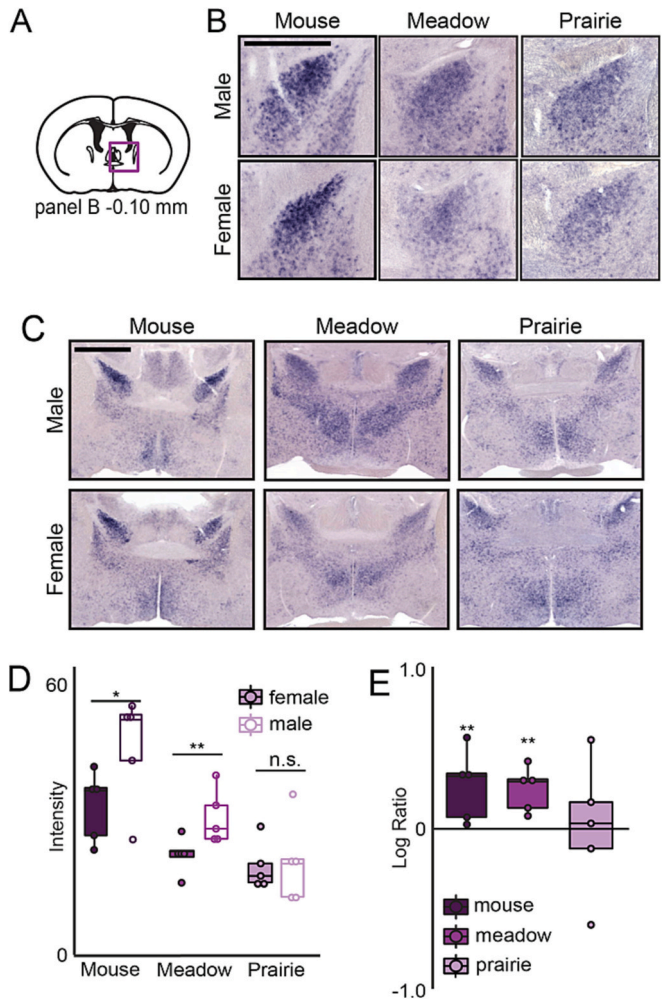

Fig. 4. Sex differences in *Ar* expression. (A) Schematic of coronal section for BNSTpr *Ar* expression and quantification. (B) ISH for *Ar* in BNSTpr. Male (top panels) and female (bottom panels) are shown for mouse, meadow vole, and prairie vole (left to right). (C) ISH for *Ar* in BNST and preoptic area (POA) of the hypothalamus. (D) Quantification of BNSTpr *Ar* expression (sum of 5 bilateral consecutive section averages) in males and females of each species (N = 5). Boxplot denotes median and 1st & 3rd quartiles, * $p < 0.05$, ** $p < 0.01$. (E) Within-batch male:female log ratios of *Ar* expression in BNSTpr. ** denotes significant difference from log ratio of zero. Scale bars = 1000 μm.

species (Fig. 5D). In contrast to a prior immunolabeling study (Ploskonka et al., 2016), we detect *Esr2* within the VMHvl of prairie voles (Fig. 5E), although our P14 timepoint is earlier than those previously assessed (P21 and P60). Staining is not present in the more lateral subdivision where we quantified a sex difference in *Esr1* (Fig. 1F,G). In meadow voles, *Esr2* signal is low and diffuse without strong enrichment in the VMHvl, in striking contrast to the other two species examined. Interestingly, we note moderate *Esr2* expression within the prairie vole arcuate nucleus, where expression is weak or absent in mice and rats (Fig. 5E) (Cao and Patisaul, 2013; Merchenthaler et al., 2004; Shughrue et al., 1997). *Esr2* is sparsely expressed in the AVPV of all three species but is very weak in prairie voles (Fig. 5F). This relative expression pattern is consistent throughout the anterior-posterior extent of the AVPV. We did not detect hippocampal *Esr2* expression in any species (Fig. 5G), shown on the same sections as MeA staining in Supplementary Fig. 5A. In the adult mouse cortex, *Esr2* is similarly sparsely expressed, with negligible expression in all inhibitory cell types (Supplementary Fig. 5B) and most excitatory cell types, with low expression in only two excitatory clusters of layer 2/3 IT PPP and IT ENTm (Supplementary Figs. 5C-G).

In the BNSTpr, we expected female *Esr2* expression to be higher than male expression, based on previous BNSTpr snRNA-seq in mouse pups (Gegenhuber et al., 2022). This pattern held true for mice (95 % CI (−0.077, −0.011); $p = 0.0052$; Fig. 6, $N = 4$ animals per sex), but we saw higher expression of *Esr2* in male meadow voles than female meadow voles (95 % CI (0.031,0.172); $p = 0.0037$; Fig. 6, N = 4 animals per sex). Prairie voles show no significant expression differences (95 % CI (−0.201,0.245); $p = 0.77$; Fig. 6, N = 4 animals per sex) between sexes in BNSTpr. In mice and prairie voles, we noted a distinctive "hole" in *Esr2* signal in males, consistent with previously described expression of an Esr2-Cre line (Zhou et al., 2023) and our prior ISH of *Esr2* in adult BNSTp (Wu and Tollkuhn, 2017). The region of BNSTpr quantification is outlined in 5 consecutive BNST sections in Supplementary Fig. 6.

3.4. Expression of gonadal hormone receptors in mouse hippocampus and cortex

With the availability of hippocampal and cortical single nucleus transcriptomic data, we examined the expression of two other gonadal hormone receptor genes, progesterone receptor (*Pgr*) and G-protein coupled estrogen receptor 1 (*Gper1*), a putative membrane receptor for estrogens (Revankar et al., 2005; Urban et al., 2023), in the mouse hippocampus and cortex. We observe moderate expression of *Pgr* within the hippocampus, largely mirroring that of *Ar*, although CA2 expression was absent (Supplementary Fig. 7 A). The pattern of *Pgr* also resembles *Ar* rather than *Esr1*, with low levels in inhibitory neurons and extensive expression in cortical and hippocampal excitatory populations (Supplementary Fig. 7B-G). In contrast, *Gper1* was barely detectable in any neuronal types examined (Supplementary Fig. 8), although expression is observed in SMC-Pericyte cell cluster 380 (Yao et al., 2021).

4. Discussion

4.1. Species differences in gonadal hormone receptor expression

Variation in steroid hormone signaling has previously been implicated as a driver of social behavior evolution (Adkins-Regan, 2012; Hoke et al., 2019; Kelly and Vitousek, 2017; Young and Crews, 1995). We observe differences in the expression patterns of gonadal hormone receptors of mice, meadow voles, and prairie voles at P14. Although with the single developmental time point investigated here, we cannot distinguish between organizational and activational roles for these receptors, we suggest that the differences we detect in the hippocampus and VMH likely influence the unique behavioral repertoires displayed by these species. The low expression of *Esr1* in both P14 and adult mouse hippocampus corroborates previous findings from adult mouse single nucleus RNA sequencing (Cembrowski et al., 2016; Yao et al., 2021) (Fig. 1D) and ISH (Lein et al., 2007), as well as earlier characterization by radiolabeled ISH in rats (Shughrue et al., 1997; Simerly et al., 1990). Previous comprehensive immunolabeling studies reported conflicting findings in mice, as well as extensive species differences between mice and rats (Merchenthaler et al., 2004; Mitra et al., 2003), however to our knowledge, no studies have simultaneously investigated both species. Prior immunolabeling studies in rats have noted sparse hippocampal labeling for ERα, which likely corresponds to the mouse cell types we identify here (Orikasa et al., 2000; Solum and Handa, 2001). Within the adult B6 mouse hippocampus, most Esr1 expression is found within two subtypes of ventral CA3 neurons, consistent with fluctuations in gene expression in the ventral hippocampus across the estrous cycle (Jaric et al., 2019). *Esr1* is also quite sparse in cortex, as demonstrated by both ISH at P14 and snRNA-seq in adults, however all clusters assigned to layer VI of the lateral entorhinal cortex show some expression.

We find that prairie voles have enriched expression of *Ar* and *Esr1* within the CA3 region of the hippocampus, which is highly interconnected with the dorsal LS in mice (Besnard and Leroy, 2022). LS-HPC connectivity is important for maintaining a cognitive spatial map

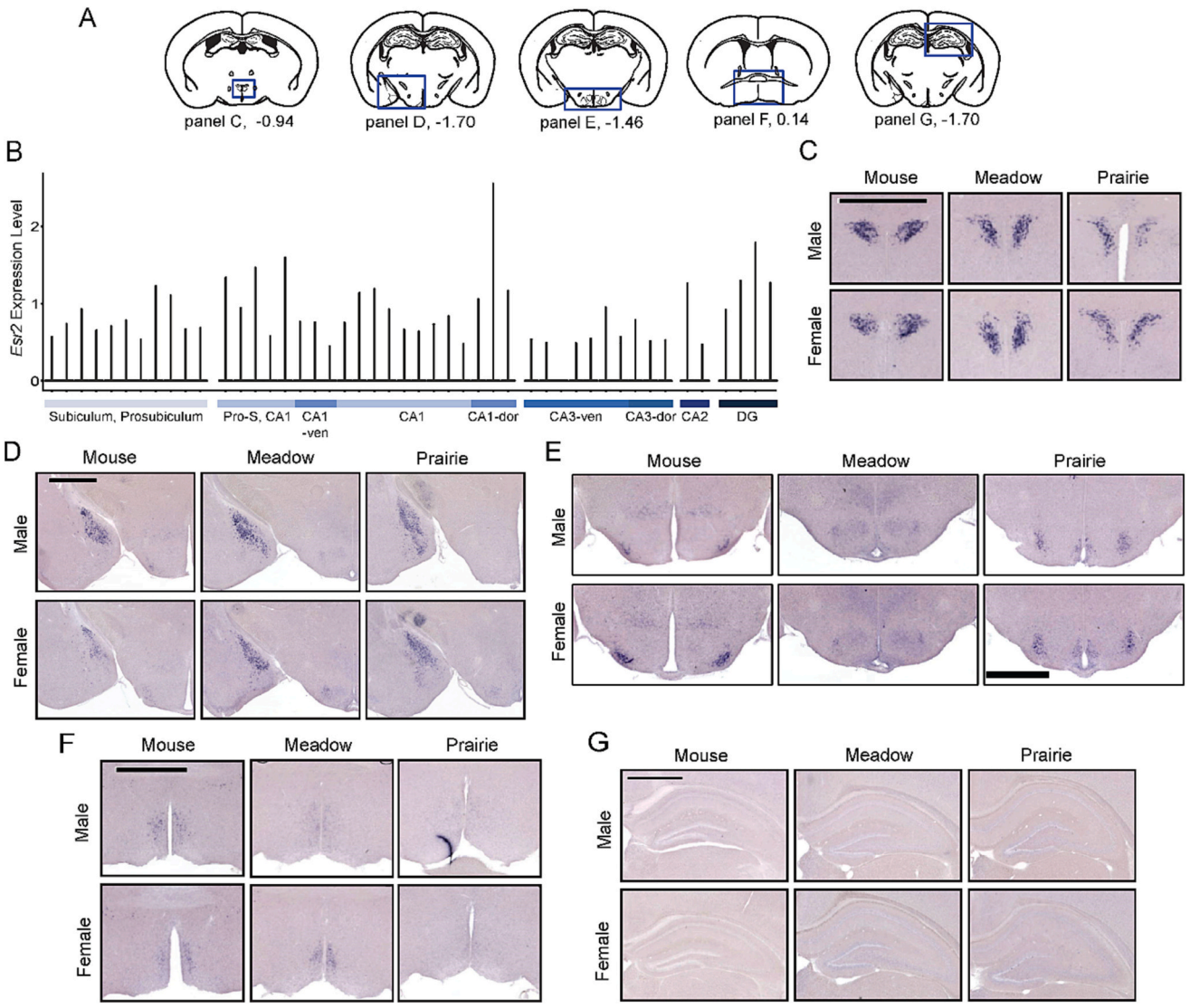

Fig. 5. Species differences in *Esr2* expression. (A) Schematics of coronal sections for the paraventricular nucleus of the hypothalamus (PVN), medial amygdala (MeA), ventromedial hypothalamus (VMH), anteroventral periventricular nucleus (AVPV), and dorsal hippocampus (dHPC). (B) Violin plot of *Esr2* expression across hippocampal cell types in adult B6 mice, organized by anatomical subregion. See Supplementary Table 2 for subclass abbreviations and cluster identity. (C-G) ISH for *Esr2* in PVN (C), MeA (D), VMH (E) AVPV (F), and dHPC (G). Male (top panels) and female (bottom panels) are shown for mouse, meadow vole, and prairie vole (left to right). Scale bars = 1000 μm.

(Tingley and Buzsáki, 2018), therefore we suggest that variation in hippocampal *Esr1* and *Ar* expression at P14 may persist into adulthood and relate to well-documented species differences in territory usage (Getz, 1961; Madison, 1980; McGuire and Getz, 1998; Ophir et al., 2008, 2012; Streatfeild et al., 2011). Prairie voles largely share and defend a territory within a bonded pair, while meadow voles of both sexes have distinct but overlapping territories. While these behaviors are not expressed at P14, the organizational effects of the sensitive neonatal period are largely complete at this stage of development and likely influence activational effects of hormones on these cell populations and the resulting expression of behavior in adulthood.

Within the CA2, *Ar* is present in mice, but not in meadow or prairie voles. This region is implicated in social memory and can promote aggression in males through disinhibition of the VMHvl via the LS (Hassan et al., 2023; Leroy et al., 2018). The absence of *Ar* in vole CA2 suggests that CA2 modulation of social memory and social aggression is independent of testosterone in voles. This hypothesis is consistent with the context-dependent selective display of aggression in both sexes of these species: meadow voles show territorial aggression during the long days of the reproductive season, while prairie voles are aggressive towards strangers only following pair bond formation (Lee and Beery, 2022). Indeed, in prairie voles, activation of the LS in pair bonded males decreases aggression towards novel conspecifics (Sailer et al., 2022). Another rodent species, Alston's singing mouse (*Scotinomys teguina*), expresses almost no AR protein in the hippocampus, with only a few cells in ventral CA3. Although males of this species show dramatic vocal displays, which are sensitive to testosterone levels, males do not attack in a standard resident-intruder aggression paradigm (Pasch et al., 2011). It will be interesting to determine how neural activity of hippocampal projections is altered in different environmental, hormonal, or social contexts in diverse rodent species.

We believe the lateral VMHvl *Esr1* population represents an expansion of the VMHvll *Cckar* neurons described in mice to be critical for female sexual behaviors (Yin et al., 2022). In prairie voles, which are induced ovulators and display less sexual dimorphism in social behavior (Carter et al., 1989; Carter and Perkeybile, 2018), this neuronal population may be more responsive to estrogens in females to facilitate the expression of female sexual behaviors after puberty and after induction

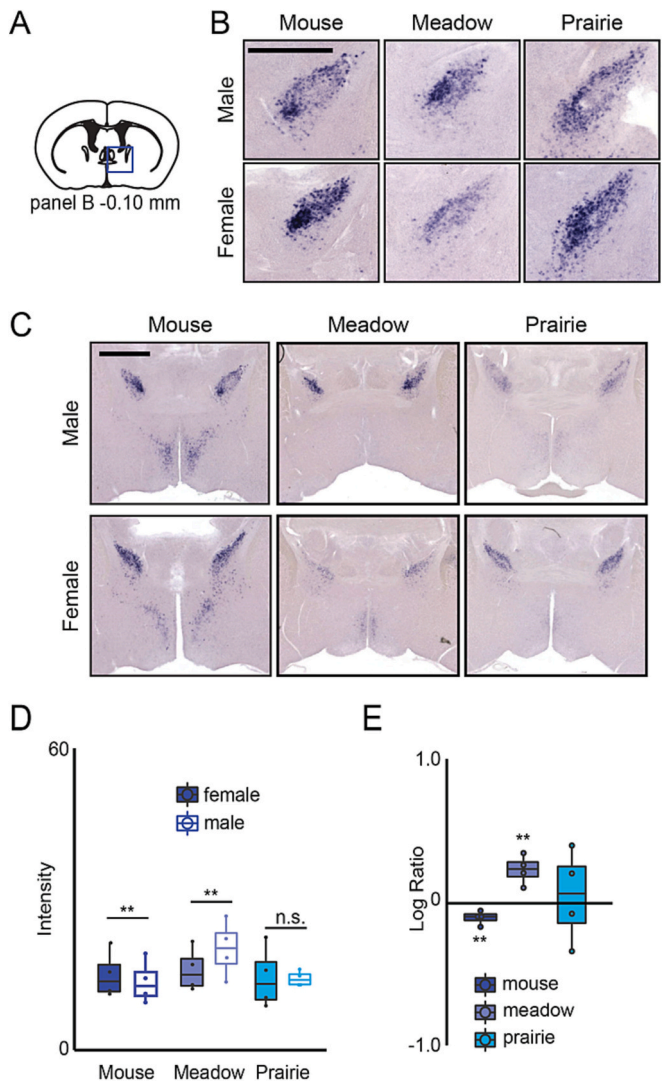

Fig. 6. Sex differences in *Esr2* expression. (A) Schematic of coronal section for BNSTpr *Esr2* expression. (B) ISH for *Esr2* in BNSTpr. Male (top panels) and female (bottom panels) are shown for mouse, meadow vole, and prairie vole (left to right). (C) ISH for *Esr2* in BNST and preoptic area (POA) of the hypothalamus. (D) Quantification of BNSTpr *Esr2* expression (sum of 5 bilateral consecutive section averages) in males and females of each species (N = 4). Boxplot denotes median and 1st & 3rd quartiles, ** $p < 0.01$. (E) Within-batch male:female log ratios of *Esr2* expression in BNSTpr. ** denotes significant difference from log ratio of zero. Scale bars = 1000 μm.

of ovulation by male-associated stimuli. Future studies could assess genes restricted to the medial (*Npy2r, Crhbp*) and lateral (*Cckar, Tac1*) VMHvl of mice to ascertain if the VMHvll in voles has a distinct identity (Hashikawa et al., 2017; Liu et al., 2021; Yin et al., 2022).

In general, *Ar* expression is more diffuse in voles than in mice. While the present study characterizes this expression at postnatal day 14, previous studies of circulating hormones in adults have shown that circulating testosterone is lower in group-housed prairie voles than in other vole species (Klein et al., 1997). In mice and rats, *Ar* expression is minimal at birth and increases dramatically over the first postnatal week, with increased expression in the BNSTpr and MPOA of males (Juntti et al., 2010; McAbee and DonCarlos, 1998). The *Ar* gene is directly regulated by ERα in the SBN (Gegenhuber et al., 2022), but is also expressed in regions with minimal ERα, such as the hippocampus and premammillary nucleus (McAbee and DonCarlos, 1998; Simerly et al., 1990).

In contrast, neural ERα expression appears in mid-gestation, when SBN regions such as the VMH and POA are still forming (reviewed in (Tobet, 2002)), with expression decreasing in males postnatally (Kelly et al., 2013; Yokosuka et al., 1997). Several studies have assessed ERα/*Esr1*, with differing results depending on age, region, species, levels of circulating hormones, or method of assessment, indicating complex regulation of this gene (Brock et al., 2015; Cao and Patisaul, 2013; Cushing et al., 2004; Cushing and Wynne-Edwards, 2006; Gegenhuber et al., 2022; Hnatczuk et al., 1994; Kelly et al., 2013; Merchenthaler et al., 2004; Mitra et al., 2003; Pérez et al., 2003; Shughrue et al., 1997; Xu et al., 2012; Yamamoto et al., 2006; Yokosuka et al., 1997). In the postnatal and adult mouse BNSTp, there are more *Esr1*-expressing cells in males, but both *Esr1* and *Esr2* are expressed at higher levels within *Esr1*+ cells in females (Gegenhuber et al., 2022). There are also reports of transient ERα/*Esr1* expression in select cortical regions (Miranda and Toran-Allerand, 1992; Pérez et al., 2003; Wilson et al., 2011; Wu and Tollkuhn, 2017; Yokosuka et al., 1995). Our results in prairie voles are in accordance with a prior study that described increased ERα immunoreactivity in prairie vole pup females at P21 but not at P8, (Yamamoto et al., 2006), possibly due to the overall increase in signal between these ages.

Our findings for *Esr2* are largely consistent with those from prior studies: within the SBN, expression is limited and appears within a subset of *Esr1*-expressing neurons (Gegenhuber et al., 2022; Merchenthaler et al., 2004; Nomura et al., 2003; Shughrue et al., 1997; Zuloaga et al., 2014). Similarly, dense expression of *Esr2* in the PVN in rats appears to be conserved in mice and voles (Ploskonka et al., 2016). Prior comprehensive characterization of ERβ-EGFP expression, using a BAC transgenic mouse line, found widespread expression at perinatal and postnatal timepoints compared to previous studies in adults and identified a sex difference in expression emerging in the AVPV at P21 (Zuloaga et al., 2014). We find surprising species differences in the VMH and arcuate nucleus, with no discernible transcript enrichment in the meadow vole VMHvl and moderate enrichment in only the prairie vole arcuate. Additional species differences may be discovered in future studies; we did not assess known sites of expression in the dorsal raphe and cerebellum (Nomura et al., 2005; Shughrue et al., 1997).

The low levels of *Esr2* reported previously in the dorsal hippocampus in rats is not seen here in mice or voles (Shughrue et al., 1997). This may be due to either species or age differences, or differences in methodological sensitivity between digoxigenin labeled and radiolabeled ISH hybridization; however, snRNA-seq data demonstrates virtually no *Esr2* transcripts in the adult mouse hippocampus, and expression within only two layer 2/3 glutamatergic cortical cell types. In contrast to the low neural expression of *Esr1*, *Esr2,* and *Gper1*, we observe that *Pgr* is widespread within hippocampus and cortical excitatory populations. Prior analyses of Pgr expression within the SBN, have found that *Pgr* is largely coexpressed with *Esr1*, and induced by estradiol so that expression is higher in adult females compared to males, but much more abundant in postnatal males compared to females (Quadros et al., 2002, 2007; Quadros and Wagner, 2008). However, expression in other regions, such as the prairie vole arcuate nucleus, is independent of gonadal hormones (Williams et al., 2013). Given the relative abundance of Pgr and Ar compared to Esr1/Esr2, it will be interesting to determine if progesterone and testosterone modulate sex differences in cortical or hippocampal neurophysiology.

4.2. Decreased sexual divergence in prairie vole BNST

The distribution of the nuclear gonadal hormone receptors, *Esr1*, *Esr2*, and *Ar*, seems to be less sexually dimorphic in socially monogamous prairie voles than socially promiscuous meadow voles and C57BL6 mice. This is consistent with the lack of both sexual dimorphism in the MPOA (Shapiro et al., 1991) and sex differences in ERβ immunoreactivity (Ploskonka et al., 2016). However, the findings of the present study contrast with previous literature in prairie voles that described intraspecies population differences in ERα expression such that the

populations exhibiting higher levels of sociality showed lower expression in the social males (i.e. more different from females) (Cushing et al., 2004; Cushing and Wynne-Edwards, 2006; Wu et al., 2011). Species differences in the relative expression of these receptors between sexes may contribute to species differences in the expression of social behaviors, particularly parental behaviors, territorial aggression, and affiliative behaviors such as pair bonding.

As with other mammalian species, prairie voles undergo a neonatal testosterone surge on the day of birth (Corbier et al., 1992; Lansing et al., 2013; Motelica-Heino et al., 1988), yet the impact of this surge on the brain and behavior appears minimal. We propose that precocial development in prairie voles relative to other rodents considered here may play an ontogenetic role in the decreased sexual dimorphism in this species (Wallen and Baum, 2002). We suggest that the development of the SBN may also be precocial, leading to a partial closure of the sensitive period for brain sexual differentiation before birth. If wiring of the SBN has substantially progressed by the time neural estradiol is produced, the effects of signaling via ERα on cell survival and gene expression could be less potent, leading to less-masculinized structures and gene expression programs in the prairie vole brain. This hypothesis is consistent with the findings of BNSTpr gonadal hormone receptor expression in the present study and prior characterization of the MPOA (Shapiro et al., 1991). Less sexual dimorphism in these structures may promote selective affiliative and prosocial behaviors in males.

This hypothesis is also consistent with brain sexual differentiation in guinea pigs, the original species in which the organizational effects of testosterone were described (Phoenix et al., 1959). Although guinea pigs are also precocial, their gestation is significantly longer than that of mice, rats, or voles, ranging from 67 to 73 days (Connolly and Resko, 1994; Goy et al., 1964). As with rhesus macaques, the critical period for guinea pig brain sexual differentiation occurs prenatally, and is specified by fetal testosterone (Resko and Roselli, 1997). Ferrets also possess dramatic MPOA sexual dimorphism which is specified by estradiol from prenatal testosterone (Park et al., 1998; Tobet et al., 1986). These earlier testosterone surges coincide with an earlier stage of brain development, resulting in more extensive sex differences. Our “intersectional” hypothesis predicts that the development timing of elevated testosterone relative to brain maturation is a key driver of anatomic and behavioral sex divergence.

4.3. Limitations and weaknesses of the present study

The present study highlights a need for species-specific brain atlases for the *Microtus* genus to define species differences in anatomy, as the comparative neuroanatomy itself is poorly characterized and poses a challenge in understanding anatomical data in these species. This study is technically limited by the use of brightfield ISH; while reproducible and presenting a uniform way to study the genes of interest, brightfield ISH does not give us cellular or subcellular resolution and can only stain for one gene at a time, which prevents co-labeling to find coexpression with other markers (such as neuronal cell type markers). Furthermore, this study gives a snapshot of brain sexual differentiation across species at postnatal day 14. While we chose this developmental time point carefully based on the documented closure of the postnatal sensitive period for brain sexual differentiation in mice and rats and critical periods for social learning in rodents, exploration of perinatal expression of hormone receptors and aromatase in voles would provide additional insight into species differences in brain sexual differentiation.

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Data availability

Data will be made available on request.

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