Activational and organizational effects of sex hormones on hippocampal inhibition

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P M conceptualized and designed the project, A H-V, A M-M, R V-R and IA performed and analyzed data. P M wrote the manuscript with the help for edition and discussion from all authors.

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Conflict of Interest

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Abstract

Peripheral and brain-produced sex hormones exert sex-specific regulation of hippocampal cognitive function. Estrogen produced by neuronal aromatase regulates inhibitory neurons (INs) and hippocampal-dependent memory in adult female mice, but not in males. How and when this sex effect is stablished and how peripheral and brain sources of estrogen interact in the control of hippocampal INs is currently unknown. Using ex-vivo electrophysiology, molecular analysis, estrous cycle monitoring and neonatal hormonal manipulations, we show evidences that suggest that neuron-derived estrogen and peripheral hormones independently exert activational effects on CA1 synaptic inhibition and perineuronal nets (PNNs) surrounding parvalbumin (PV)-expressing INs. Before puberty, aromatase is expressed in PV INs and regulates synaptic inhibition in female but not in male mice. Neonatal testosterone administration abolished the effect of brain-derived estrogen on adult female synaptic inhibition and disrupted brain-derived estrogen regulation of PV IN PNNs. Our results suggest that sex differences in brain-derived estrogen effects on CA1 inhibition are established by organizational effects of neonatal gonadal hormones and highlight the role of INs as mediators of the sexual differentiation of the hippocampus.

Highlights

- Increased coverage of CA1 PV INs by perineuronal nets (PNNs) in proestrus.
- No apparent estrous cycle related changes in CA1 synaptic inhibition.
- Aromatase protein is expressed in male and female PV neurons before puberty.
- Brain-derived estrogen regulates CA1 synaptic inhibition in females not in males.
- Neonatal testosterone disrupts estrogen effects on adult female hippocampal CA1 synaptic inhibition and PNNs.

Introduction

In the adult brain, sex hormones regulate neuronal function and influence cognition through sex-specific actions in the hippocampus (Fleischer and Frick, 2023), a brain structure involved in learning, memory and spatial navigation. Sex-specific hormonal effects support basic neuronal mechanisms underlying cognitive function (Azcoitia et al., 2022; Yagi and Galea, 2019) and have been linked to the sex bias in the prevalence of neurodevelopment disorders, such as Autism Spectrum Disorders and Intellectual Disability (Bölte et al., 2023). Moreover, changes in sex hormone levels and reproductive function interact with aging in promoting cognitive deficits (Zárate et al., 2017). Despite the relevance to understand cognition in the healthy, aging and diseased brain, how and when sex effects are implemented in the hippocampus is not fully understood.

Estrogen regulates the function of hippocampal inhibitory neurons (INs) (Huang and Woolley, 2012; Murphy et al., 1998), that release the neurotransmitter ɣ-amino butyric acid (GABA) and dictate the temporal coordination of excitatory neuronal activity underlying hippocampal cognitive functions (Klausberger and Somogyi, 2008). Estrogen reduces inhibitory neurotransmission onto CA1 excitatory pyramidal neurons (Huang and Woolley, 2012; Tabatadze et al., 2015), a process in which local production of estrogen by neuronal aromatase is critically involved. Neuron-derived estrogen (neuroestrogen) limits the coverage of hippocampal CA1 parvalbumin (PV)-expressing INs by perineuronal nets (PNNs), extracellular proteoglycan structures that enwrap PV INs and regulate excitability and plasticity of this IN type (Hernández-Vivanco et al., 2022). Importantly, akin to previous results on excitatory synaptic function (Kretz et al., 2004; Wang et al., 2018), neuroestrogen regulation of synaptic inhibition and PV-INs PNNs is only detected in female mice hippocampus and not in males (Hernández-Vivanco et al., 2022). The mechanisms that give rise to sex differences in the regulation of hippocampal PV INs by neuroestrogen are currently unknown.

Sex differences in mammalian brain arise from the different sex chromosome complement (XX and XY) of male and female neurons and from the action of local and peripheral produced sex hormones (McCarthy et al., 2012). The organizational - activational theory of brain sexual differentiation posits that hormonal production by late embryonic and neonatal testis trigger sex-specific genetic programs (Gegenhuber and Tollkuhn, 2020) with enduring consequences in connectivity and physiology of neuronal networks (organizational effects). In addition, activational effects of sex hormones released by the gonads after puberty exert transient and reversible actions in a sex specific manner (Arnold, 2009). Using genetically modified mice to break the link between gonadal and genetic sex, we have previously shown that neuroestrogen reduces CA1 synaptic inhibition

and PV INs PNNs coverage in gonadal female mice with XX or XY sex chromosome complement (Hernández-Vivanco et al., 2022). These results suggest that female-specific neuroestrogen actions on hippocampal inhibition and PV INs PNNs are independent of the genetic sex of the brain and raise the alternative possibility that sex effects are determined by adult (activational) or neonatal (organizational) actions of gonadal hormones.

Here we investigated the origin of sex differences in neuroestrogen regulation of CA1 synaptic inhibition and hippocampal PV INs using ex-vivo electrophysiology, molecular analysis, and estrous cycling monitoring. We first tested whether estrous cycle-related activational effects of ovarian hormones regulate CA1 synaptic inhibition, PV INs PNNs and aromatase expression. We then determined whether neuroestrogen regulates CA1 synaptic inhibition before functional maturation of the gonads and used neonatal hormonal manipulations to test organizational effects on CA1 synaptic inhibition. Our results show activational effects of ovarian hormones on CA1 PV INs and unveil organizational effects of neonatal gonadal hormones on hippocampal inhibition.

Results

Estrous cycle regulation of CA1 synaptic inhibition and PV INs.

In order to investigate the effects of ovarian hormones on CA1 synaptic inhibition and INs, we determined spontaneous Inhibitory Post-Synaptic Currents (sIPSCs) in CA1 excitatory pyramidal neurons and perineuronal net (PNN) coverage of PV INs in the CA1 area in female mice in different stages of the estrous cycle. We chose those parameters because sIPSCs frequency and PV INs PNNs coverage are both increased by pharmacological reduction of neuroestrogen synthesis with aromatase inhibitors in female mice (Hernández-Vivanco et al., 2022). Additionally, we determined the influence of the estrous cycle on the expression of aromatase in CA1 PV INs (Fig. 1A). At 10-12 weeks of age, adult female mice were assigned to diestrus (low estrogen) and proestrus (high estrogen) groups by performing vaginal cytologies (Fig. 1B and Methods section).

We recorded sIPSCs on visually identified CA1 pyramidal neurons with intact network activity in acutely prepared brain slices from female mice processed in the morning of proestrus or diestrus. In contrast to the previously observed regulation by neuroestrogen (Hernández-Vivanco et al., 2022), the frequency and amplitude of sIPSCs did not show apparent differences between proestrus and diestrus female mice (Fig. 1C).

We used histological sections from diestrus or proestrus female mice to determine PNN coverage of CA1 PV INs (Wisteria floribunda agglutinin staining, see Methods) and aromatase expression in

PV INs with specific antibodies. WFA intensity around PV INs in proestrus was higher compared with diestrus (Fig. 1D, E, WFA in PV). In contrast, diestrus and proestrus female mice showed similar levels of aromatase expression in PV INs (Fig. 1F Arom in PV).

These results show that the estrous cycle influence PV INs PNN coverage but do not apparently modify CA1 synaptic inhibition in pyramidal neurons and aromatase expression in female mice PV INs. Together with the previously observed effects of neuroestrogen, these results suggest that ovarian hormones and neuroestrogen exert different and independent activational effects on CA1 synaptic inhibition and PV INs PNNs.

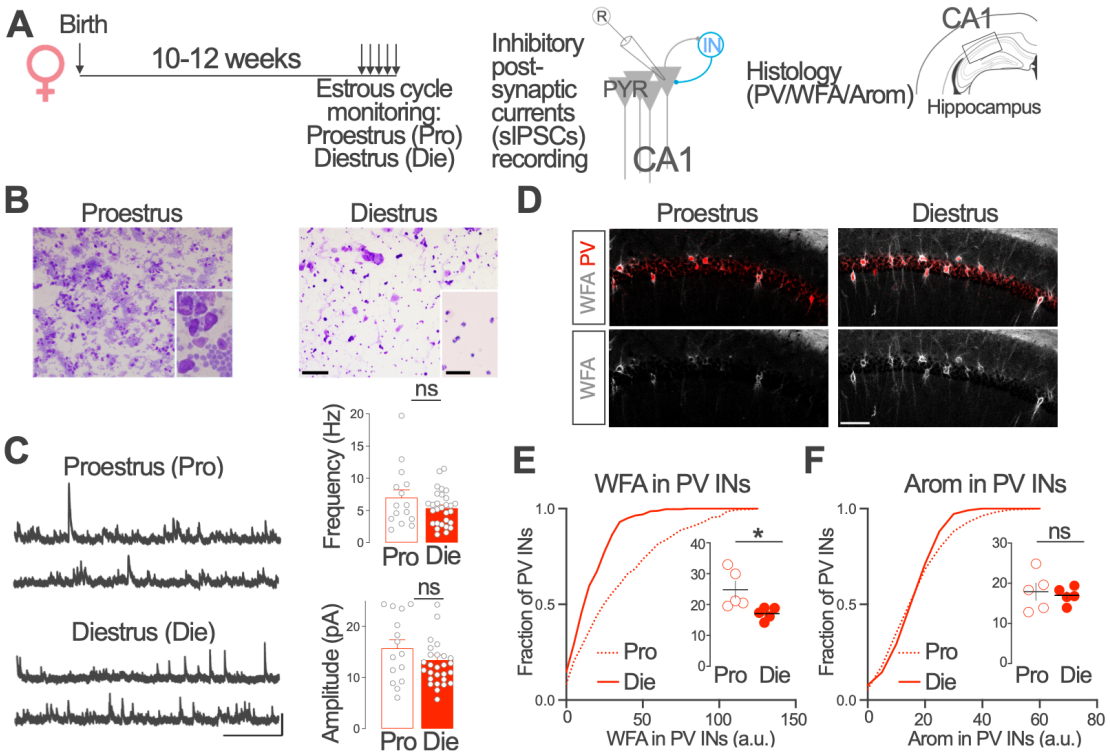

Figure 1. *Estrous cycle related changes in CA1 synaptic inhibition and PNNs surrounding PV INs.*
A. Estrous cycle was monitored using vaginal cytologies in 10-12 weeks old female mice. In the morning of diestrus or proestrus, mice were processed for Spontaneous Inhibitory Post-Synaptic Currents (sIPSCs) recordings in acutely prepared brain slices or histological analysis in the CA1 area of the hippocampus (approximate location, grey area). **B**. Representative images (10 and 40 times magnification, inset) of cresyl violet-stained vaginal smears assign to proestrus (left) and diestrus (right). Scale bars: 100 µm (25 µm inset). **C**. Representative recordings of sIPSCs in Proestrus (Pro) and diestrus (Die) female mice. Scale bar: 50 pA, 1 s. Graphs represent group data. Frequency, Two-tailed Mann Whitney test, U = 187, p = 0.46. Amplitude, Unpaired two tailed t-test, t (42) = 1.27, p = 0.21. n = 15, 29 neurons from 3-4 mice per group. **D.** Representative image of simultaneous parvalbumin (PV, red) immunohistochemical detection and WFA staining of PNNs (grey) in dorsal hippocampus CA1 region of an adult female mice in proestrus (left) and diestrus (right). Single channel image of WFA staining is represented in grey in the lower part of the panel. Scale bar: 100 µm. **E.** Group data of WFA staining intensities in PV-INs. Cumulative frequency distribution of individual values per PV IN and mean values per mice are shown. Unpaired two tailed t-test, t (8) = 2.67, p = 0.03. **F.** Group data of aromatase staining intensities in PV INs. Cumulative frequency distribution of individual values per PV IN and mean values per mice are shown. Unpaired two tailed t-test, t (8) = 0.39, p = 0.7. n = 5 mice per group. Graphs represent mean +/- SEM (columns and bars) and individual values (recorded neurons, grey circles in C mice; circles in F). Graphs in E and F represent cumulative distribution of WFA and aromatase staining in individual PV INs.* p < 0.05; ns p > 0.05.

Aromatase expression and neuroestrogen production in PV INs before puberty.

The previous results suggest that neuroestrogen affects hippocampal INs independently of the function of the adult ovaries. To further test this idea, we investigated neuroestrogen production by hippocampal PV INs before puberty, i.e., before the start of gonadal hormone production in male and female mice. We used immunohistochemistry to detect aromatase protein in CA1 PV INs at postnatal day (PND) 21, before puberty onset in mice (Fig. 1A).

Aromatase expression was observed in CA1 region of PND21 male and female mice (Fig. 2B). Aromatase immunoreactive cells were found in different layers, mainly in the pyramidal and orients strata (Fig. 2B). Simultaneous localization of aromatase and PV in CA1 area of male and female mice showed aromatase expression in this IN type in male and female mice (Fig. 2B). Aromatase expression levels in CA1 PV INs of PND 21 male and female mice did not show significant differences (Fig. 2C). WFA staining indicated that aromatase-expressing PV INs were surrounded by PNNs in male and female CA1 region at PND 21 (Fig. 2C).

These results show that aromatase is expressed in PV INs before the start of sex hormone production by adult gonads in male and female mice and suggest prepubertal synthesis of neuroestrogen by hippocampal PV INs with PNN coverage.

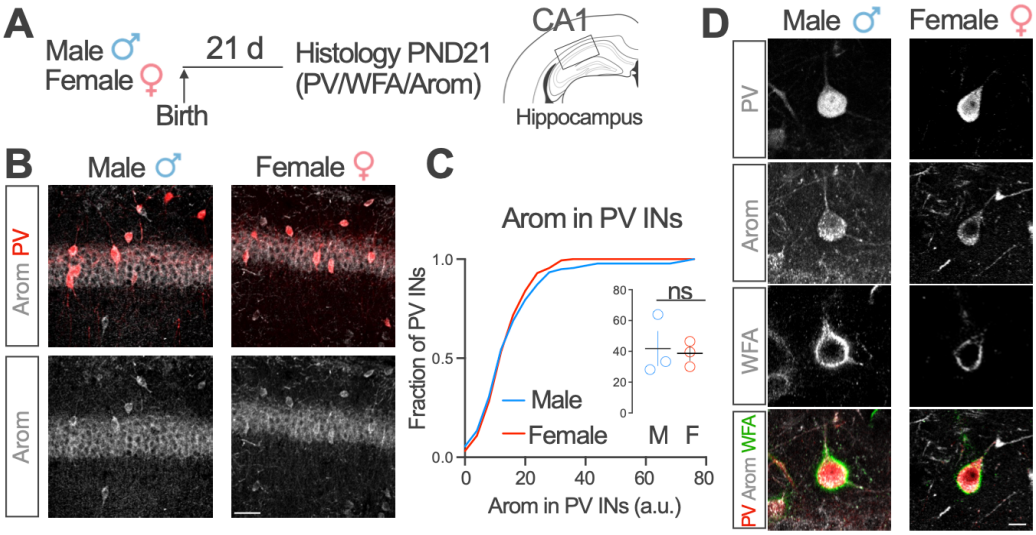

Figure 2. Aromatase expression in CA1 PV INs in prepubertal mice.
A. Male and female mice were processed for immunohistochemistry at post-natal day 21. **B**. Representative immunofluorescence confocal microscopy images of aromatase (grey) and parvalbumin (red, upper panels) expression in the CA1 region of male (left) and female (right) hippocampus at 21 days of age. Single channel image of aromatase staining is represented in grey scale in the lower part of the panel. Scale bar: 50 µm. **C.** Quantification of aromatase expression level in M and F hippocampus. Group data of aromatase staining intensities in PV-INs. Cumulative frequency distribution of individual values per PV IN and mean values per mice are shown. Two-tailed Mann Whitney test, U = 4, $p > 0.99$. $n = 3$ mice per group. **D**. Representative immunofluorescence confocal microscopy images of simultaneous detection of parvalbumin (PV), aromatase (Arom) and and WFA staining of PNNs (WFA) in the CA1 region of male and female hippocampus at 21 days of age.
Graphs represent mean +/- SEM and individual values. ns $p > 0.05$.

Estrogen regulation of CA1 synaptic inhibition before puberty.

The presence of aromatase in PV IN at PND 21 suggest a functional impact of neuroestrogen on synaptic inhibition onto CA1 excitatory pyramidal neurons in prepubertal mice. To test this idea, we treated male and female mice with aromatase blocker Letrozole between PND21 and PND25 (0.5 mg/kg, one daily intraperitoneal injection during 5 days, Arom Block, Fig. 3A). Letrozole crosses the blood-brain barrier (Zhou et al., 2010) and has been previously shown to increase synaptic inhibition in adult female mice hippocampus (Hernández-Vivanco et al., 2022). On PND25, at the end of the treatment period, we performed patch-clamp recordings of sIPSCs from CA1 pyramidal neurons in acutely-prepared brain slices.

In female mice, aromatase blockade increased sIPSCs frequency in CA1 pyramidal neurons compared with vehicle treated mice (Fig. 3B, C). We observed no significant change in the amplitude of sIPSC in female mice (Fig. 3B C). In contrast, in male mice, aromatase blockade did not produce apparent changes in sIPSC frequency and amplitude compared with vehicle treated male mice (Fig. 3B, C).

These results show that aromatase inhibition before puberty increases synaptic inhibition onto CA1 excitatory pyramidal neurons in female mice but not in male mice.

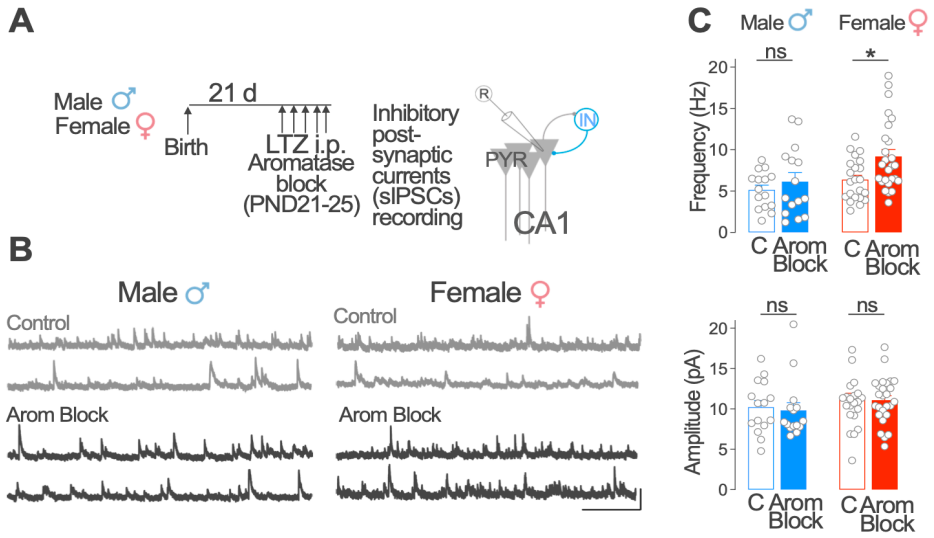

Figure 3. *Aromatase regulation of CA1 synaptic inhibition in prepubertal male and female mice*.
A. Post-Natal Day (PND) 21 male and female mice received daily intraperitoneal (i.p.) injections of the aromatase blocker letrozole (LTZ) or vehicle (C) for 5 days. Spontaneous Inhibitory Post-Synaptic Currents (sIPSCs) were recorded from CA1 pyramidal (PYR) neurons in acutely prepared slices at PND25. **B**. Representative sIPSCs recordings from control (C, grey) and letrozole (Arom Block, black) treated male (left) and female (right) mice. Scale bar: 50 pA, 1 s. **C**. Group data from sIPSCs recordings. Frequency, two-way ANOVA, C/Arom Block F (1, 74) = 5.48, p = 0.02, Male/Female F (1, 74) = 6.9, p < 0.01. Bonferroni comparison tests, Male C vs Arom Block p = 0.87; Female C vs Arom Block p = 0.01. Amplitude, two-way ANOVA, C/Arom Block F (1, 74) = 0.09, p = 0.77, Male/Female F (1, 74) = 1.97, p < 0.16. Bonferroni comparison tests, Male C vs Arom Block p > 0.99; Female C vs Arom Block p > 0.99. Males, n = 15 neurons from 3 mice per group; females, n = 22 (Control), 27 (Arom Block) neurons from 3 mice per group.
Graphs represent mean +/- SEM (columns and bars) and individual values (recorded neurons, grey circles). * p < 0.05; ns p > 0.05.

Neonatal testosterone impact on neuroestrogen regulation of CA1 synaptic inhibition and PV INs PNNs.

The observed female-specific effects of neuroestrogen in prepubertal mice suggest that sex-specific neuroestrogen regulation of synaptic inhibition originates from early-life organizational effects of neonatal hormones. To test this idea, we treated neonatal female mice pups with testosterone propionate (100 µg in 50 µl of sesame oil, one daily injection on PND 1, 8 and 15) to mimic the male-specific perinatal testosterone surge. This treatment has been previously shown to masculinize behavior dependent on aromatase expressing neurons in different regions of the female brain (Wu et al., 2009). Ten weeks after testosterone or vehicle neonatal treatment, we tested neuroestrogen regulation of synaptic inhibition and PV INs PNNs by treating adult mice at 12 weeks of age with the aromatase specific inhibitor letrozole (0.5 mg/kg, one daily intraperitoneal injection during 5 days, Arom Block, Fig. 4A). We determined sIPSCs in CA1 pyramidal neurons and PV INs PNNs coverage using electrophysiological recordings and immunohistochemistry, respectively.

In line with previous results in adult mice (Hernández-Vivanco et al., 2022) and prepubertal mice (Fig. 3), aromatase blockade increased sIPSC frequency in neonatal vehicle treated female mice. While neonatal testosterone treatment did not produce significant sIPSCs frequency changes when compared with vehicle treated mice, it completely prevented the effect of aromatase blocker letrozole on sIPSC frequency (Fig. 4B). We observed no significant differences in the amplitude of sIPSCs between the experimental groups (Fig. 4B).

Cumulative frequency distribution analysis of WFA staining intensities showed that aromatase blockade increases the intensity of WFA staining surrounding CA1 PV INs in neonatal vehicle treated mice. In contrast, aromatase inhibition reduced WFA staining in PV INs of neonatal testosterone-treated mice.

These results show that neonatal testosterone treatment in female mice prevents neuroestrogen effects on synaptic inhibition in CA1 pyramidal neurons and disrupts the regulation of CA1 PV INs PNNs. These results strongly suggest organizational effects of neonatal hormones in neuroestrogen regulation of CA1 synaptic inhibition and CA1 PV INs.

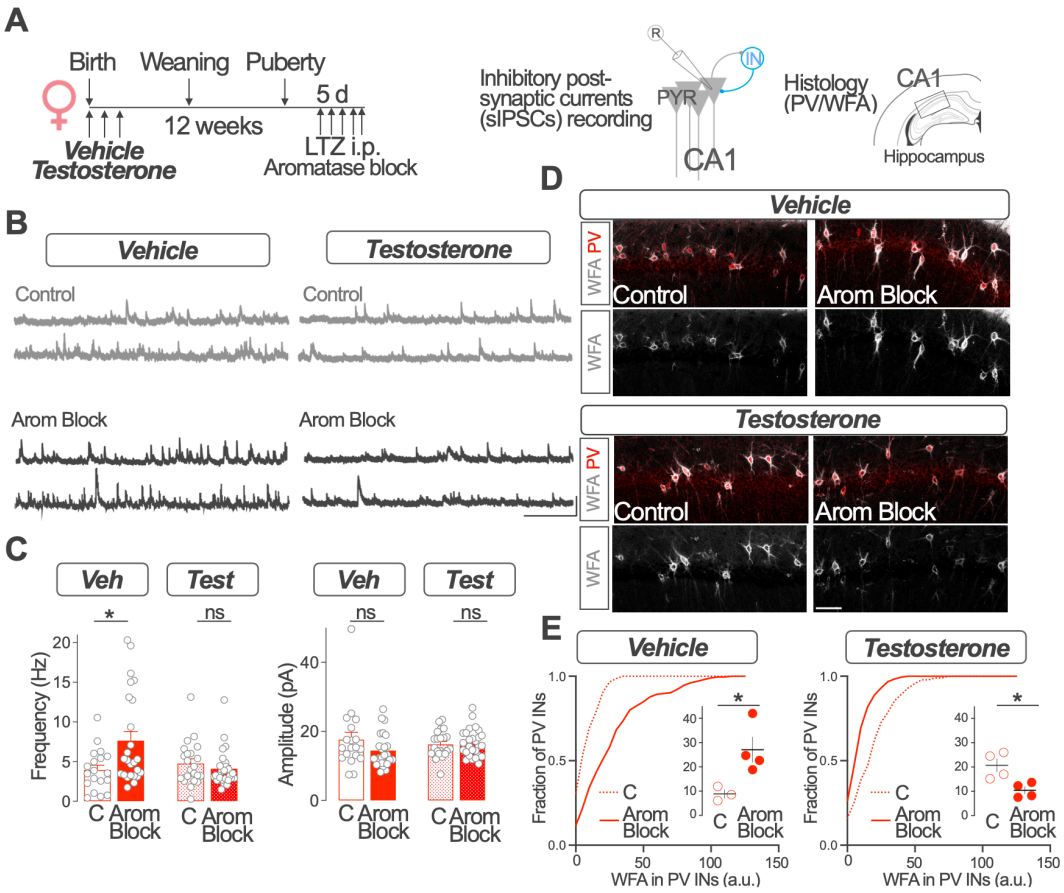

Figure 4. *Neonatal testosterone effects on the regulation of hippocampal inhibition*.

A. Female mice pups received subcutaneous injections of testosterone propionate (100 µg) or vehicle (sesame oil) on post-natal days 1, 7 and 15, weaned and raised to young adulthood (12 weeks). Adult mice were then treated with the aromatase blocker letrozole (LTZ) or vehicle (C) for 5 days and processed for Spontaneous Inhibitory Post-Synaptic Currents (sIPSCs) recordings or histological analysis.

B. Representative sIPSCs recordings from control (C, grey) and letrozole (Arom Block, black) treated female mice with neonatal vehicle (left) and testosterone (right) treatment. Scale bar: 50 pA, 1 s.

C. Group data from sIPSCs recordings. Frequency, two-way ANOVA, C/Arom Block F (1, 90) = 3.97, p = 0.05, Veh/Test F (1, 90) = 3.2, p = 0.07. Bonferroni comparison tests, Veh C vs Arom Block p = 0.002; Test C vs Arom Block p = 0.78. Amplitude, two-way ANOVA, C/Arom Block F (1, 90) = 1.82, p = 0.18, Veh/Test F (1, 90) = 0.02, p = 0.89. Bonferroni comparison tests, Veh C vs Arom Block p = 0.14; Test C vs Arom Block p = 0.99. Vehicle, control, n = 19 neurons, 3 mice; Arom Block n = 25 neurons, 4 mice; Testosterone, control, n = 22 neurons, 4 mice; Arom Block n = 28 neurons, 4 mice.

D. Representative image of simultaneous parvalbumin (PV, red) immunohistochemical detection and WFA staining (grey) in the CA1 region of neonatal vehicle (upper panels) or testosterone (lower panels) treated female mice which received daily intraperitoneal (i.p.) injections of the aromatase blocker letrozole (LTZ, Arom Block, right panels) or vehicle (Control, C, left panels). Single channel image of WFA staining is represented in grey scale in the lower part of the panels. Scale bar: 100 µm.

E. Group data of WFA staining intensities in PV-INs for neonatal vehicle (left) or testosterone (right) treated female mice. Cumulative frequency distribution of individual values per PV IN and mean values per mice are shown in each case. Vehicle, C vs Arom Block, unpaired two tailed t-test, t (5) = 2.91, p = 0.03. Testosterone, C vs Arom Block, unpaired two tailed t-test, t (6) = 3.32, p = 0.02. Vehicle, control, n = 3 mice; Arom Block n = 4 mice; Testosterone, control, n = 4 mice; Arom Block n = 4 mice.

Graphs represent mean +/- SEM (columns and bars) and individual values (recorded neurons, grey circles in C mice; circles in E). Graphs in E represent cumulative distribution of WFA staining for individual PV INs.* $p < 0.05$; ns $p > 0.05$.

Discussion

Excitatory neurons are targets for activational actions of sex hormones in the hippocampus (Taxier et al., 2020). Our results show estrous cycle related changes in hippocampal INs, suggesting activational effects of gonadal hormones also in inhibitory neurons. In particular, females in proestrus, a stage of the estrous cycle associated with a rise in circulating estradiol and progesterone concentrations, show increased PNN neuronal coverage in the dorsal CA1 hippocampus. In this region, PNNs mostly surround a prominent type of PV INs, PV-expressing basket cells (Yamada and Jinno, 2015). Since PNNs regulate physiology and plasticity of PV INs (Fawcett et al., 2019), our results suggest that cyclic ovarian production of sex hormones may affect dorsal hippocampal function through the regulation of this IN type. The well documented involvement of hippocampal PV INs PNNs in learning and memory processes (Favuzzi et al., 2017; Ramsaran et al., 2023) suggest that PNNs modulation across the estrous cycle may be related with the effects of peripheral hormones on memory (Taxier et al., 2020) and on differential hippocampal engagement in spatial tasks (Korol et al., 2004). Additionally, fluctuation of PNNs across the estrous cycle may be responsible for the behavioral response to non-cognitive aspects such as stress or aging (Laham et al., 2022).

We have previously reported that, in adult female mice, reduced neuroestrogen levels increase PV INs PNN coverage (Hernández-Vivanco et al., 2022), see also Fig. 4E). In contrast, here we observed that high circulating estrogen levels during proestrus are associated to increased PNN coverage of PV INs. The differential effect on CA1 PV INs PNNs suggests that neuron-derived estrogen and cycling gonadal-derived ovarian hormones regulate CA1 PV INs PNNs through different mechanisms. Estrous cycle regulation of PNNs may involve the actions of other ovarian derived hormones such as progesterone (Laham et al., 2022). Moreover, estrous cycle may affect the molecular composition of chondroitin sulphate proteoglycans of PNNs, which has strong consequences on neuronal plasticity (Yang et al., 2021). In contrast with neuroestrogen functional effects limiting CA1 synaptic inhibition (Hernández-Vivanco et al., 2022), estrous cycle is not reflected in apparent changes in IPSCs frequency in CA1 pyramidal neurons, suggesting that brain-derived estrogen and ovarian hormones effect diverge also in their functional effects in CA1 synaptic inhibition and PV INs.

The contribution and functional consequences of local synthesis of estrogen by hippocampal INs before sexual maturity is currently unknown. Our results suggest that aromatase is expressed in CA1 PV INs in the male and female hippocampus at post-natal day 21 in male and female mice. Thus, CA1 PV INs may contribute to local synthesis of estrogen before the start of sex hormone production by adult gonads. Interestingly, our results also show that systemic pharmacological

blockade of aromatase activity in prepubertal mice has functional effects on synaptic inhibition of CA1 pyramidal neurons. Aromatase inhibition increases sIPSC frequency in CA1 pyramidal neurons of prepubertal female but not male mice. Since juvenile gonadal hormone production remains at very low levels, these results strongly suggest female-specific effects of brain-produced neuroestrogen in the physiology of hippocampal INs in prepubertal mice. Importantly, this sex effect is detected before functional maturation of gonads, again suggesting that neuroestrogen and ovarian hormones independently regulate the function of CA1 INs. Through the regulation of inhibitory signaling and PV INs PNNs in the prepubertal hippocampus, neuroestrogen may promote the refinement of network activity (Cossart and Khazipov, 2022), control the closure of inhibition-dependent critical periods for brain plasticity (Miranda et al., 2022) and promote in this way the formation of precise memories (Ramsaran et al., 2023). Moreover, neuroestrogen actions in the prepubertal brain may have a functional impact in the development and maturation of hippocampal INs that takes places during this stage of life, affecting processes such as programmed cell death and synaptogenesis (Lim et al., 2018; Wong and Marín, 2019).

Our results evidence functional effects of sex hormones in the neonatal and preadolescent mice brain. During this period, sex hormones and their receptors are at their highest levels, prior to gradually decline to adult levels (Turano et al., 2019). Early exposure to testosterone in female mice pups renders adult CA1 inhibition insensitive to neuroestrogen and disrupts PV INs PNNs regulation. This suggest that neonatal production of testosterone by testes in male mice triggers organizational effects on CA1 synaptic inhibition and PV INs. Since aromatase expression is detected before puberty in PV INs in male mice, neonatal testosterone effects in this neuron type may be mediated by its local aromatization to estrogen (Wu et al., 2009). Organizational effects of neonatal testosterone have been reported in hippocampal excitatory neurons and have been proposed to explain sex differences in metabotropic glutamate receptor signaling in hippocampal neurons in vitro (Meitzen et al., 2012). Thus, neonatal hormones may coordinately organize excitatory and inhibitory hippocampal neurons to promote sex specific regulation of excitatory / inhibitory balance in developing networks.

The early life period coincides with the maturation and functional integration of different hippocampal neuronal types and the emergence and refinement of spatially-tuned activity characteristic of CA1 place cells (Wills et al., 2010) and hippocampal network activity synchrony (Farooq and Dragoi, 2019). The critical role of hippocampal INs in controlling spatial coding (Valero et al., 2022) and oscillations (Klausberger and Somogyi, 2008) raises the possibility of functional consequences of organizational actions of sex hormones in hippocampal processes known to be important for episodic memory. Moreover, by impacting neuronal communication, organizational

actions of neonatal hormones may support functional network maturation and prevent deviations from normal neurodevelopmental trajectories with enduring consequences.

Methods

Animals. All experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee of the Cajal Institute and by local veterinary authorities (Comunidad de Madrid). Group housed male and female mice in the CD1 genetic background were maintained in a 12 h light/dark cycle, 20–22 °C, 45–65% humidity and with unlimited access to food. All animals were obtained from the animal facility of the Cajal Institute. Age and sex of the animals is described for each experiment in the corresponding figure and legend.

Estrous cycle monitoring. Estrous cycle was monitored by vaginal cytologies performed between 7 and 10 am. A vaginal lavage with 75 µl saline solution was collected using a P200 pipette with a rounded tip. The lavage was repeated several times to ensure efficient cell sampling and placed in a gelatin-coated microscope slide. After drying, the sample was stained with cresyl violet (0.1 %) and imaged in an optical microscope using 10x and 40x objectives. The estrous cycle stage was determined according to the relative presence of epithelial cells (nucleated and cornified) and leukocytes.

Reagents and hormonal treatments. Letrozole (Tocris) was dissolved in DMSO to 12.5 mg/ml, further dissolved in saline solution to 62.5 µg/ml and administered at a dose of 0.5 mg/kg in intraperitoneal (i.p.) injections of 8 ml/kg. Testosterone propionate (Sigma) was dissolved in sesame oil by overnight magnetic stirring at a concentration of 2 mg/ml. Female pups received interscapular subcutaneous injections (50 µl) using a 25 gauge needle.

Slice electrophysiology. To prepare acute slices for electrophysiological recordings, brains were quickly removed and coronal slices (300 µm) containing the dorsal hippocampus were obtained with a vibratome (4°C) in a solution containing: 234 mM sucrose, 11 mM glucose, 26 mM $NaHCO_3$, 2.5 mM KCl, 1.25 mM NaH_2PO_4, 10 mM $MgSO_4$, and mM 0.5 $CaCl_2$ (equilibrated with 95% O2–5% CO2). Recordings were obtained at 30-32°C from CA1 stratum pyramidale neurons visually identified using infrared video microscopy in oxygenated artificial cerebrospinal fluid containing 126 mM NaCl, 26 mM $NaHCO_3$, 2.5 mM KCl, 1.25 mM NaH_2PO_4, 2 mM $MgSO_4$, 2 mM $CaCl_2$ and 10 mM glucose (pH 7.4). Patch-clamp electrodes contained intracellular solution composed of: 127 mM Cesium methanesulfonate, 2 mM CsCl, 10 mM HEPES, 5 mM EGTA, 4 mM MgATP, and 4 mM QX-314 bromide, pH 7.3 adjusted with CsOH (290 mOsm). GABA receptors mediated inhibitory spontaneous (sIPSCs) were registered by clamping neurons at 0 mV. Signals were amplified using a Multiclamp 700B patch-clamp amplifier and digitized using a Digidata 1550B (Axon Instruments,

USA), sampled at 20 kHz, filtered at 10 kHz, and stored on a PC using Clampex 10.7 (Axon Instrumenst). Series resistance was monitored by a voltage pulse in every recorded cell and compared between experimental groups to discard effects due to recording conditions. IPSC were analyzed using pClamp (Axon Instruments) and a custom written software (Detector, courtesy J. R. Huguenard, Stanford University), as previously described (Manseau et al., 2010). Briefly, individual events were detected with a threshold-triggered process from a differentiated copy of the real trace. For each cell, the detection criteria (threshold and duration of trigger for detection) were adjusted to ignore slow membrane fluctuations and electric noise while allowing maximal discrimination of sIPSCs. Detection frames were regularly inspected visually to ensure that the detector was working properly.

Tissue processing and immunohistochemistry. Mice were injected with a lethal dose of pentobarbital (150mg/kg) and perfused transcardiacally with cold PBS and 4% paraformaldehyde solution. Brains were extracted and submerged in fixative for 4 hours at 4º C. Coronal 40 µm thick sections containing dorsal hippocampus were blocked in PBS 0.3% BSA , 5% normal goat serum (NGS) and 0.3 %Triton X-100 followed by overnight incubation in PBS, 5% NGS and 0.3 %Triton X-100 with primary antibody: parvalbumin (guinea pig polyclonal, code GP42, Swant) and aromatase (1:1000, in-house production, described and validated in (Yague et al., 2006)). Biotinylated Wisteria Floribunda Lectin (Vector Laboratories) was incubated in the same conditions as primary antibodies. After 3x15 minutes wash in PBST at room temperature, slices were incubated with 1:500 Alexa-conjugated secondary antibodies and Steptavidin (Alexa-Fluor 488, 555, Abcam) to reveal primary antibodies and biotynilated WFA, respectively. After 3 more step of washing in PBST, slices were mounted and covered on microscope slides using DAPI containing mounting medium.

Image analysis. Images were obtained with a Leica SP5 confocal microscope (LEICA LAS AF software) using 20x or 40x objectives and 405, 488, 561 nm laser excitation wavelengths. 1024x1024 images with a resolution of 1.3-2.6 pixel/µm, at 2-4 µm step size were collected. Manually depicted ROIs delimiting CA1 PV neurons were used to determine fluorescence intensity in other channels (aromatase). For quantification of WFA staining, a lineal ROI surrounding PV neurons (3,8 µm width) was used. Mean pixel intensity in closed and lineal ROIs was determined in equally thresholded images.

Statistical analysis. All values are given in mean ±SEM, except when noted. Standard t tests were performed to compare Gaussian distributions while Mann-Whitney tests were used for non-gaussian distributions. One- or two-way ANOVA followed by Bonferroni´s post hoc test were used when noted. For all tests, we adopted an alpha level of 0.05 to assess statistical significance. Statistical analysis was performed using Prism (Graphpad software).

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