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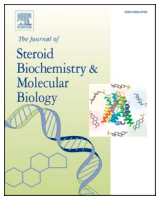

# Sex chromosome complement interacts with gonadal hormones in determining regional-specific neuroactive steroid levels in plasma, hippocampus, and hypothalamus. A study using the four core genotype mouse model

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ABSTRACT

An important aspect of the neuromodulatory and neuroprotective actions exerted by neuroactive steroids is that they are sex-specific, as determined by the sexually dimorphic levels of these molecules in plasma and the nervous tissue. Thus, the identification of the factors that generate the sex-dimorphic levels of neuroactive steroids may be crucial from a neuroprotectant perspective. The main driver for sex determination in mammals is the SRY gene and the subsequent presence of a specific gonad: testes for males and ovaries for females, thus producing hormonal compounds, primarily androgens and estrogens, respectively. Nowadays, it is well established that despite the relevance of gonads, other factors control sexual features, and, among them, sex chromosome complement is highly relevant. In this study, neuroactive steroids were evaluated by liquid chromatography-tandem mass spectrometry in the hypothalamus, the hippocampus, and plasma of the four core genotype mouse model, to determine the relative contribution of sex chromosome complement and gonads in determining their sex dimorphic levels. The data obtained reveal that although gonads are the main contributing factor for sex differences in neuroactive steroid levels, the levels of some neuroactive steroids, including testosterone, are also influenced in brain and plasma by tissue-specific actions of sex chromosomes. The data presented here adds a new piece to the puzzle of steroid level regulation, which may be useful in designing sex-specific neuroprotective approaches to pathological conditions affecting the nervous system.

## 1. Introduction

The term neuroactive steroids (i.e., steroid molecules regulating the nervous functions) includes both steroid hormones synthesized by endocrine glands, such as testis, ovary, and adrenal glands, and neurosteroids synthesized by the nervous system [1,2]. Examples of neuroactive steroids are represented by the first steroid formed from cholesterol, pregnenolone (PREG), its metabolites dehydroepiandrosterone (DHEA), progesterone (PROG), testosterone (T), and the further metabolites, dihydrotestosterone (DHT) and 3alpha-diol (3α-diol) in the case of T and dihydroprogesterone (DHP), allopregnanolone (ALLO), and isoallopregnanolone (ISOALLO) in the case of PROG.

Several new concepts have emerged in the field of neuroactive steroids during the last decade. For instance, (i) neuroactive steroid levels in plasma do not exactly reflect what occurs in the nervous system, (ii) different brain areas express different neuroactive steroid levels, and (iii) all these aspects are different in males and females. In addition, a close relationship between gonadal steroid hormones and nervous levels of neuroactive steroids has been reported. Indeed, gonadectomy affects the levels of neuroactive steroids in the nervous system [3]. These

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**Table 1**
Pregnenolone (PREG), progesterone (PROG), dihydroprogesterone (DHP), allopregnanolone (ALLO) isoallopregnanolone (ISOALLO), dehydroepiandrosterone (DHEA), testosterone (T), dihydrotestosterone (DHT) and 5α-androstane-3α, 17β-diol (3α-diol) levels in plasma, hippocampus and hypothalamus of (XX/Sry-) and (XY/Sry+) control groups. Data are expressed as pg/μL in case of plasma and pg/mg in case of brain tissues and are the mean ± SEM. u.d.l. = under detection limit. Detection limits were 0.05 pg/μL or pg/mg for DHEA, DHT, 3α-diol; 0.1 pg/μL or pg/mg for ALLO and ISOALLO. n =5-8 animals for each experimental group. The unpaired Student's t-test or Mann-Whitney were used for statistical analysis depending on the data (see materials and methods). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs (XX/Sry-) female control group.

| | PREG | | PROG | DHP | | ALLO | | ISOALLO | | DHEA | | T | | DHT | | 3α-diol | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | XX/ Sry- | XY/ Sry+ | XX/Sry- | XY/ Sry+ | XX/Sry- | XY/ Sry+ | XX/ Sry- | XY/ Sry+ | XX/ Sry- | XY/ Sry+ | XX/ Sry- | XY/ Sry+ | XX/ Sry- | XY/ Sry+ | XX/ Sry- | XY/ Sry+ | XX/ Sry- | XY/ Sry+ |
| PLASMA | 0.409 ± 0.044 | 3.222 ± 0.545 ** | 18.760 ± 5.214 | 0.247 ± 0.083 * | 0.750 ± 0.162 | 1.606 ± 0.442 | 1.530 ± 0.397 | u.d.l. ± 0.000 *** | u.d.l. ± 0.000 | 0.691 ± 0.167 ** | 0.096 ± 0.024 | 0.138 ± 0.026 | 0.043 ± 0.012 | 2.526 ± 0.599 *** | u.d.l. ± 0.000 | 0.273 ± 0.138 | u.d.l. ± 0.000 | 0.527 ± 0.144** |
| HIPPOCAMPUS | 7.443 ± 0.789 | 11.550 ± 1.228 * | 24.260 ± 7.793 | 0.994 ± 0.420 ** | 18.100 ± 6.359 | 6.966 ± 1.604 | 3.756 ± 1.401 | 7.836 ± 2.493 | 1.275 ± 0.343 | u.d.l. ± 0.000 ** | u.d.l. ± 0.000 | 0.170 ± 0.088 | 0.244 ± 0.096 | 1.565 ± 0.323 *** | u.d.l. ± 0.000 | 0.879 ± 0.462 | u.d.l. ± 0.000 | u.d.l. ± 0.000 |
| HYPOTHALAMUS | 6.482 ± 0.783 | 7.648 ± 1.779 | 18.400 ± 4.494 | 0.416 ± 0.224 ** | 10.820 ± 2.127 | 3.910 ± 1.629 * | u.d.l. ± 0.000 | 9.059 ± 0.839 *** | 1.428 ± 0.582 | 0.821 ± 0.442 | u.d.l. ± 0.000 | 0.952 ± 0.464 * | 0.068 ± 0.031 | 3.244 ± 0.657 *** | u.d.l. ± 0.000 | 1.927 ± 0.765 ** | u.d.l. ± 0.000 | 1.392 ± 0.710 * |

changes show regional specificity between different brain areas and do not necessarily reflect changes in plasma steroid levels. Moreover, the effects of gonadectomy are different in males and females and depend on the duration of gonadal hormone deprivation (i.e., short or long-term castration) [3]. Recent results indicate that, at least in some cases, the sex-specific effects of long-term gonadectomy are associated with alterations in the expression of neurosteroidogenic machinery [4]. However, the expression of steroidogenic enzymes, such as aromatase, is also influenced by sex chromosome complement [5,6]. This type of study takes advantage of the four core genotype (FCG) model. Indeed, this mouse model combines a spontaneous deletion of the sex-determining region Y (*Sry* gene) from the Y chromosome ($Y^{-}$) with the re-insertion of a functional *Sry* transgene onto an autosome [7–9]. Therefore, XY animals that also have the *Sry* gene (animals Sry+) in autosomes develop testes. In addition, XX animals with the *Sry* gene in an autosome also develop testes. Therefore, with this experimental model, the following four animal groups may be obtained: (1) XX Sry-, normal females (two X chromosomes and ovaries). Equivalent to normal XX animals; (2) XY Sry+, normal males because they have testes (due to the *Sry* gene in an autosome) and have one X chromosome and one Y chromosome. Equivalent to normal XY animals; (3) XX Sry+, these animals are phenotypically males because they have testes due to the *Sry* gene in an autosome, but genetically females because they have two XX chromosomes; (4) XY Sry-, these animals are phenotypically females: they have ovaries because they lack the *Sry* gene. However, they are genetically males because they have one X chromosome and one Y chromosome.

Therefore, to evaluate the effects of gonads and/or sex chromosomes on neuroactive steroid levels in these animals, we assessed the levels of PREG, PROG, DHEA, DHP, ALLO, ISOALLO, T, DHT and 3α-diol by liquid chromatography-tandem mass spectrometry in the plasma, hippocampus, and hypothalamus of the four experimental groups mentioned above. In the case of female animals, the diestrus phase was considered.

## 2. Material and methods

### 2.1. Animals

57 Four Core Genotype (FCG, B6.Cg-Tg(Sry)2Ei Srydl1Rlb/ArnoJ) mice from our in-house colony were used for the experiments, specifically 21 females (XX Sry-), 9 females (XX Sry+), 11 males (XY Sry+) and 16 males (XY Sry-). Animals were kept on a 12:12-h light–dark cycle and received food and water ad libitum. At the age of 3 weeks, FCG animals were genotyped by RT-PCR detection of *Sry* and *Ssty* (located in the Y chromosome) gene transcripts, and animals with the same type of gonadal organs were housed together independently to the chromosomal endowment. Animals were handled in accordance with the guidelines published in the "NIH Guide for the care and use of laboratory animals", the principles presented in the "Guidelines for the Use of Animals in Neuroscience Research" by the Society for Neuroscience, and following the European Union (2010/63/UE) and the Spanish legislation (L6/2013; RD53/2013). Experimental procedures were approved by our Institutional Animal Use and Care Committee (Comité de Ética de Experimentación Animal del Instituto Cajal) and by the Consejería del Medio Ambiente y Territorio (Comunidad de Madrid, PROEX 059.5/21). Special care was taken to minimize animal suffering and to reduce the number of animals used to the minimum necessary.

## 3. Experimental procedure

At three-month-old of age, the animals were euthanized by decapitation and the hypothalami and hippocampi were meticulously dissected under a magnifying lens. Each animal's whole blood was individually harvested and deposited into a sterile, heparinized tube. Plasma was subsequently extracted by the isolation of the aqueous phase following centrifugation at 1000 rpm for 10 minutes. The samples were

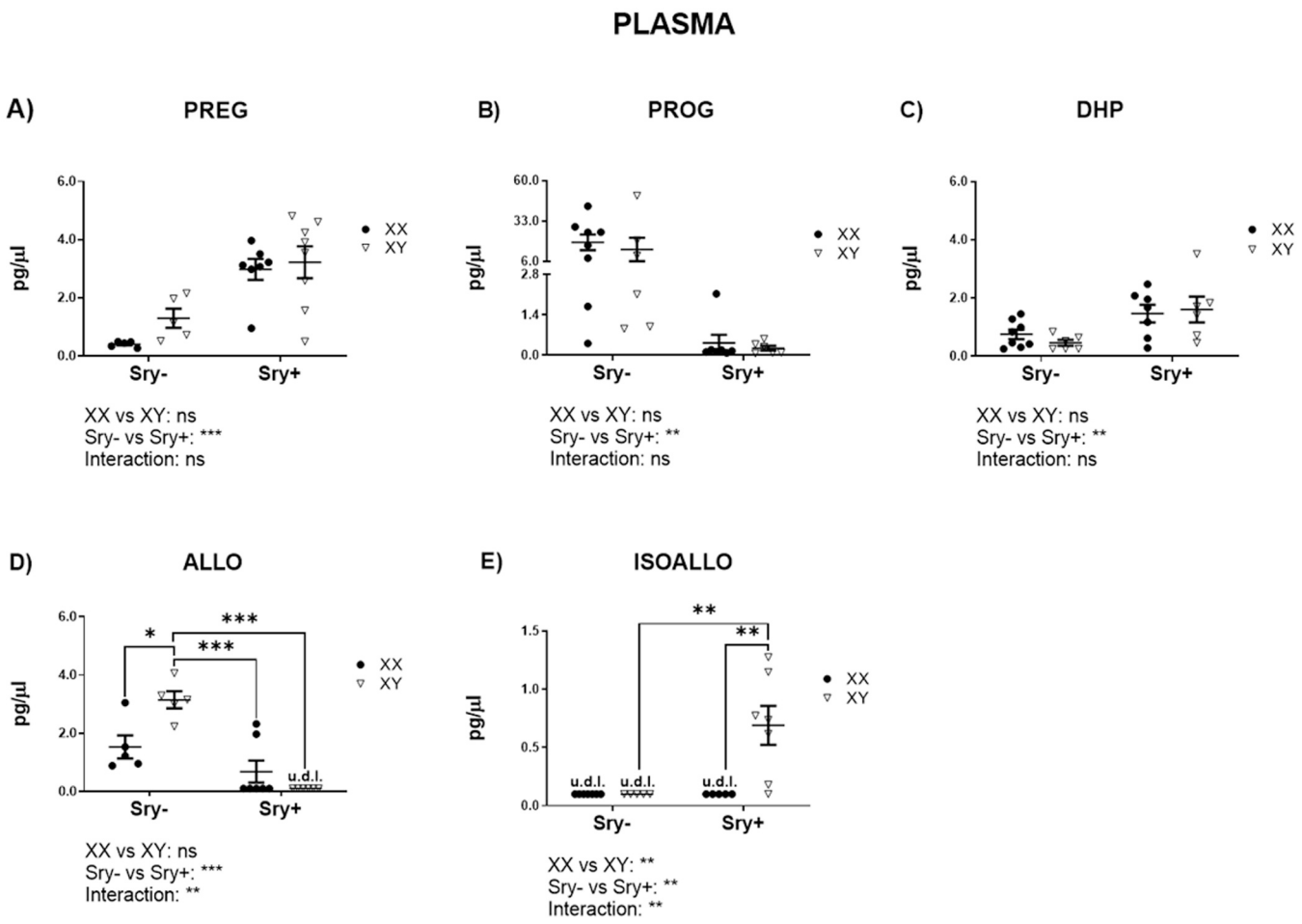


**Fig. 1.** Plasma levels assessed by LC-MS/MS of four core genotype animals. The panels represent the levels of (**A**) pregnenolone (PREG); (**B**) progesterone (PROG); (**C**) dihydroprogesterone (DHP); (**D**) allopregnanolone (ALLO); (**E**) isoallopregnanolone (ISOALLO). Data are expressed as pg/µL and are the mean ± SEM. u.d.l. = under detection limit. Detection limit was 0.1 pg/µL for ALLO and ISOALLO. n = 5–8 animals for each experimental group. The two-way ANOVA was used for statistical analysis followed by the multiple comparison Tukey post-hoc test. Statistically significant differences of sex chromosme complement (XX vs XY), gonadal phenotype (Sry- vs Sry+) and the interaction of sex chromosome complement with gonadal phenotype (interaction) are indicated as follows: * $p < 0.05$, ** $p < 0.01$, ***, $p < 0.001$, ns, not significant difference.

immediately flash-frozen using dry ice and securely stored at −80°C until they were ready for additional processing. Female mice (both XX Sry- and XY Sry-), were sacrificed during the diestrus 2 phase. The determination of the estrous cycle phase was performed by analyzing vaginal smears [10].

### *3.1. Liquid chromatography–tandem mass spectrometry analysis (LC-MS/MS)*

Neuroactive steroid levels, such as PREG, PROG, DHP, ALLO, ISO-ALLO, DHEA, T, DHT and 3α-diol were assessed by LC-MS/MS in plasma and brain tissue of FCG mice as previously described [11]. For tissue samples, the hippocampus and the hpothalamus were selected because previous studies in rats have shown the existence of sex differences in neuroactive steroid levels in both brain regions [3,11].

Brain tissues and plasma from the 57 animals were pooled to process at least 250–300 µL of plasma, 45–60 mg of hippocampus and 20 mg of hypothalamus in order to obtain the necessary amount of tissue for the analisys and to achieve a minimum of n=5 for each experimental group.

17β-Estradiol-2,3,4-$^{13}C_3$ ($^{13}C_3$-17β-E, 2 ng/ sample), progesterone-2,3,4,20,25-$^{13}C_5$ ($^{13}C_5$–PROG, 0.4 ng/sample) and pregnenolone-20,21-$^{13}C_2$-16,16 $D_2$ ($^{13}C_2$ $D_2$–PREG, 10 ng/ sample) were used as internal standards and added to all samples. Brain samples were homogenized using a Tissue Lyser (QIAGEN, Italy), in ice-cold methanol/acetic acid 1% and plasma samples were diluted in acetonitrile. All samples were extracted by an overnight precipitation at 4°C and then purified using C18 SPE cartridges (HyperSep C18 SPE Columns 500 mg 3 mL; Microcolumn, Milano, Italy).

The analysis was performed using liquid chromatography supplied by Surveyor LC Pump Plus and Surveyor Autosampler Plus (Thermo Fisher Scientific MA, USA) with a linear ion trap-mass spectrometer LTQ (Thermo Fisher Scientific MA, USA), operated in positive atmospheric pressure chemical ionization (APCI+). The chromatographic separation was achieved with a Hypersil Gold column C18 (100 × 2.1 mm, 3 µm; Thermo Fisher Scientific MA, USA) maintained at 40 °C. The mobile phases consisted of 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in methanol (mobile phase B). Gradient elution was as follows: 0–1.50 min 70% A, 30% B; 1.50–2.00 min 55% A, 45%B; 2.00–3.00 min 55% A, 45% B; 3.00–35.00 min linear gradient to 36% A, 64% B; 35.00–40.00 min 25% A, 75% B; 41.00–45.00 min 1% A, 99% B; 45.00–45.20 min 70% A, 30% B and 45.40–55.00 min equilibrated with 70% A and 30% B. 25 µL sample was injected at a flowrate of 0.250 mL/min. The divert valve was set at 0–8 min to waste, 8–45 min to source, and 45–55 min to waste. The injector needle was washed with methanol/water 1:1 (v/v). Quantitative analysis of steroids was performed using the calibration curves freshly prepared and the internal standard method ($^{13}C_3$-17β-E for T and DHEA, $^{13}C_5$–PROG for PROG, DHT and 3α-diol, $^{13}C_2$ $D_2$–PREG for PREG, DHP, ALLO and ISOALLO). LC-MS/MS

**Table 2**
Two-way ANOVA analysis of pregnenolone (PREG), progesterone (PROG), dihydroprogesterone (DHP), allopregnanolone (ALLO) isoallopregnanolone (ISOALLO), dehydroepiandrosterone (DHEA), testosterone (T), dihydrotestosterone (DHT) and 5α-androstane-3α, 17β-diol (3α-diol) levels in plasma (PL), hippocampus (HIPP) and hypothalamus (HYP) of FCG mice groups, with chromosome and Sry transgene onto an autosome as two independent variables. Dfn = degree of freedom for the numerator of the F ratio. Dfd = degrees of freedom for denominator of the F ratio.

| PL | PREG | | PROG | | DHP | | ALLO | | ISOALLO | | DHEA | | T | | DHT | | 3α-diol | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | F (DFn, DFd) | P value | F (DFn, DFd) | P value | F (DFn, DFd) | P value | F (DFn, DFd) | P value | F (DFn, DFd) | P value | F (DFn, DFd) | P value | F (DFn, DFd) | P value | F (DFn, DFd) | P value | F (DFn, DFd) | P value |
| Interaction | F (1, 21) = 0.54 | 0.4685 | F (1, 23) =0.24 | 0.6268 | F (1, 23) = 0.62 | 0.4390 | F (1, 19) = 11.90 | **0.0027** | F (1, 20) = 8.66 | **0.0080** | F (1, 21) = 1.48 | 0.2377 | F (1, 23) = 5.82 | **0.0242** | F (1, 20) = 0.34 | 0.5649 | F (1, 24) = 0.44 | 0.5107 |
| Sry- vs Sry+ | F (1, 21) = 26.12 | **<0.0001** | F (1, 23) = 11.72 | **0.0023** | F (1, 23) = 11.50 | **0.0025** | F (1, 19) = 37.18 | **<0.0001** | F (1, 20) = 8.66 | **0.0080** | F (1, 21) = 0.61 | 0.4446 | F (1, 23) = 13.11 | **0.0014** | F (1, 20) = 15.11 | **0.0009** | F (1, 24) = 33.40 | **<0.0001** |
| XX vs XY | F (1, 21) = 1.67 | 0.2098 | F (1, 23) = 0.28 | 0.6007 | F (1, 23) = 0.08 | 0.7853 | F (1, 19) = 2.62 | 0.1220 | F (1, 20) = 8.66 | **0.0080** | F (1, 21) = 0.25 | 0.6209 | F (1, 23) = 5.78 | **0.0247** | F (1, 20) = 0.34 | 0.5649 | F (1, 24) = 0.44 | 0.5107 |
| **HIPP** | **PREG** | | **PROG** | | **DHP** | | **ALLO** | | **ISOALLO** | | **DHEA** | | **T** | | **DHT** | | **3α-diol** | |
| | **F (DFn, DFd)** | **P value** | **F (DFn, DFd)** | **P value** | **F (DFn, DFd)** | **P value** | **F (DFn, DFd)** | **P value** | **F (DFn, DFd)** | **P value** | **F (DFn, DFd)** | **P value** | **F (DFn, DFd)** | **P value** | **F (DFn, DFd)** | **P value** | **F (DFn, DFd)** | **P value** |
| Interaction | F (1, 21) = 14.40 | **0.0011** | F (1, 24) = 0.18 | 0.6775 | F (1, 24) = 0.01 | 0.9456 | F (1, 17) = 2.26 | 0.1512 | F (1, 24) = 1.63 | 0.2144 | F (1, 21) = 0.43 | 0.5172 | F (1, 24) = 11.18 | **0.0027** | F (1, 23) = 4.53 | **0.0443** | / | / |
| Sry- vs Sry+ | F (1, 21) = 7.93 | **0.0103** | F (1, 24) = 16.80 | **0.0004** | F (1, 24) = 7.87 | **0.0098** | F (1, 17) = 1.01 | 0.3296 | F (1, 24) = 3.96 | 0.0582 | F (1, 21) = 4.73 | **0.0412** | F (1, 24) = 18.76 | **0.0002** | F (1, 23) = 11.03 | **0.0030** | / | / |
| XX vs XY | F (1, 21) = 1.00 | 0.3286 | F (1, 24) = 0.21 | 0.6499 | F (1, 24) = 0.43 | 0.5177 | F (1, 17) = 0.19 | 0.6677 | F (1, 24) = 0.03 | 0.8698 | F (1, 21) = 0.43 | 0.5172 | F (1, 24) = 11.36 | **0.0025** | F (1, 23) = 0.39 | 0.5360 | / | / |
| **HYP** | **PREG** | | **PROG** | | **DHP** | | **ALLO** | | **ISOALLO** | | **DHEA** | | **T** | | **DHT** | | **3α-diol** | |
| | **F (DFn, DFd)** | **P value** | **F (DFn, DFd)** | **P value** | **F (DFn, DFd)** | **P value** | **F (DFn, DFd)** | **P value** | **F (DFn, DFd)** | **P value** | **F (DFn, DFd)** | **P value** | **F (DFn, DFd)** | **P value** | **F (DFn, DFd)** | **P value** | **F (DFn, DFd)** | **P value** |
| Interaction | F (1, 18) = 2.17 | 0.1583 | F (1, 21) = 2.42 | 0.1345 | F (1, 19) = 3.44 | 0.0792 | F (1, 16) = 2.22 | 0.1558 | F (1, 21) = 1.73 | 0.2022 | F (1, 20) = 0.81 | 0.3783 | F (1, 21) = 9.47 | **0.0057** | F (1, 21) = 0.38 | 0.5437 | F (1, 19) = 4.68 | **0.0435** |
| Sry- vs Sry+ | F (1, 18) = 0.54 | 0.4738 | F (1, 21) = 34.75 | **<0.0001** | F (1, 19) = 2.39 | 0.1389 | F (1, 16) = 18.01 | **0.0006** | F (1, 21) = 1.09 | 0.3093 | F (1, 20) = 2.21 | 0.1527 | F (1, 21) = 24.49 | **<0.0001** | F (1, 21) = 5.82 | **0.0251** | F (1, 19) = 4.68 | **0.0435** |
| XX vs XY | F (1, 18) = 0.14 | 0.7165 | F (1, 21) = 0.52 | 0.4793 | F (1, 19) = 0.17 | 0.6808 | F (1, 16) = 5.39 | **0.0338** | F (1, 21) = 1.50 | 0.2343 | F (1, 20) = 0.12 | 0.7314 | F (1, 21) = 10.43 | **0.0040** | F (1, 21) = 0.21 | 0.6509 | F (1, 19) = 4.68 | **0.0435** |

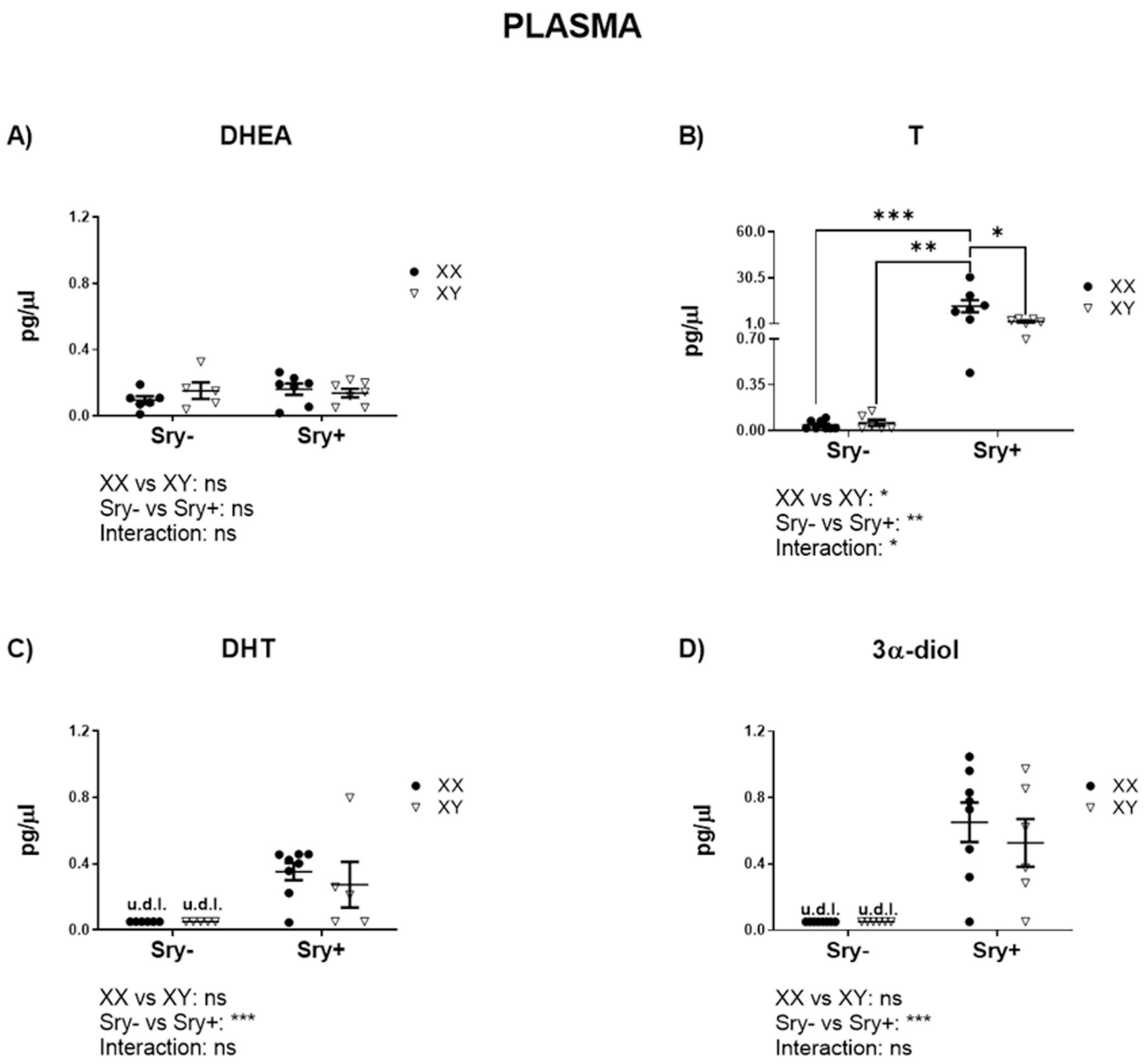


**Fig. 2.** Plasma levels assessed by LC-MS/MS of four core genotype animals. The panels represent the levels of (**A**) dehydroepiandrosterone (DHEA); (**B**) testosterone (T); (**C**) dihydrotestosterone (DHT); (**D**) 5α-androstane-3α, 17β-diol (3α-diol). Data are expressed as pg/µl and are the mean ± SEM. u.d.l. = under detection limit. Detection limit was 0.05 pg/µL for DHT and 3α-diol. n = 5–8 animals for each experimental group. The two-way ANOVA was used for statistical analysis followed by the multiple comparison Tukey post-hoc test. Statistically significant differences of sex chromosme complement (XX vs XY), gonadal phenotype (Sry- vs Sry+) and the interaction of sex chromosome complement with gonadal phenotype (interaction) are indicated as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns, not significant difference.

data were evaluated using the software Excalibur® release 2.0 SR2 (Thermo Fisher Scientific MA, USA). Detection limits were 0.02 pg/µL or pg/mg for T, 0.05 pg/µL or pg/mg for PREG, PROG, 3α-diol, DHEA, DHT; 0.1 pg/µL or pg/mg for ALLO and ISOALLO; 0.25 pg/µL or pg/mg for DHP.

### 3.2. Statistical analysis

LC-MS/MS data were analyzed using a two-way analysis of variance (ANOVA), with chromosome and *Sry* transgene onto an autosome as two independent variables, followed by Tukey post-hoc test. After checking the distribution with the Kolmogorov-Smirnov test ($p < 0.05$ was considered significant), statistical differences between female (XX Sry-) and male (XY Sry+) control mice were assessed by Student's t-test. All analyses were performed using GraphPad PRISM (La Jolla, CA, USA) (version 7.2).

## 4. Results

The levels of different steroids were analyzed by LC-MS/MS in the plasma, hippocampus, and hypothalamus of FCG mice. As a first step, the results in female (XX Sry-) and male (XY Sry+) control mice were compared to identify sex differences in steroid levels. As shown in Table 1, statistical analysis performed by unpaired Student's t-test showed significantly high levels of PREG in the plasma and hippocampus of male rats. In contrast, the levels of its first metabolite, PROG, were

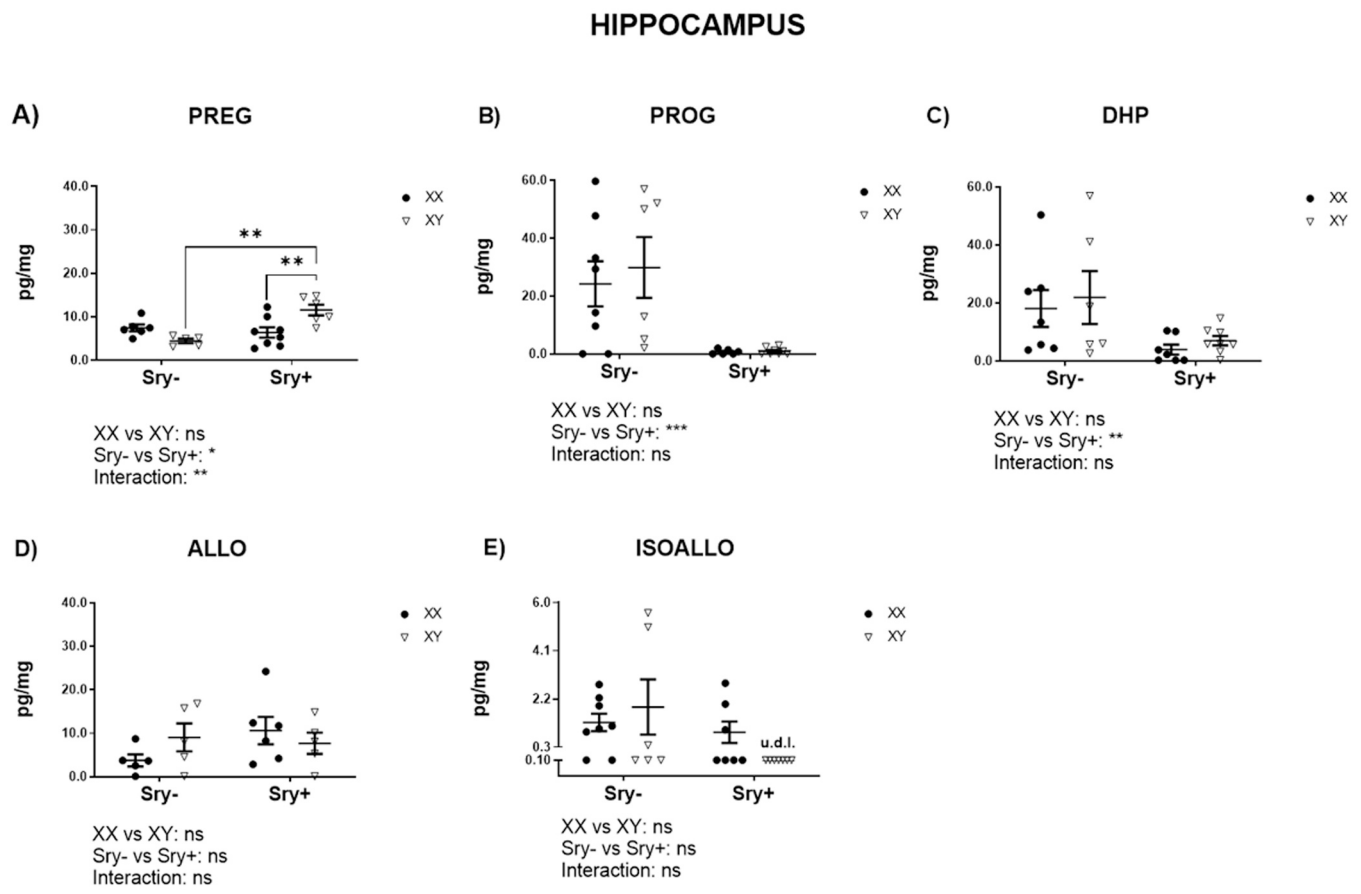


**Fig. 3.** Hippocampal levels assessed by LC-MS/MS of four core genotype animals. The panels represent the levels of (**A**) pregnenolone (PREG); (**B**) progesterone (PROG); (**C**) dihydroprogesterone (DHP); (**D**) allopregnanolone (ALLO); (**E**) isoallopregnanolone (ISOALLO). Data are expressed as pg/mg and are the mean ± SEM. u.d.l. = under detection limit. Detection limit was 0.1 pg/mg for ISOALLO. n = 5–8 animals for each experimental group. n = 5–8 animals for each experimental group. The two-way ANOVA was used for statistical analysis followed by the multiple comparison Tukey post-hoc test. Statistically significant differences of sex chromosme complement (XX vs XY), gonadal phenotype (Sry- vs Sry+) and the interaction of sex chromosome complement with gonadal phenotype (interaction) are indicated as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns, not significant difference.

higher in the plasma, hippocampus, and hypothalamus of female animals. PROG metabolites showed peculiar sex differences. Indeed, while DHP levels were higher in the female hypothalamus, ALLO levels were higher in the male hypothalamus and in female plasma. ISOALLO levels were not sex dimorphic in the hypothalamus but were higher in the plasma of male mice and higher in the hippocampus of female mice. The levels of DHEA and its metabolites were generally higher in males than in females. In particular, DHEA was significantly higher in male hypothalamus, T in plasma, hippocampus and hypothalamus, DHT in hypothalamus and 3α-diol in plasma and hypothalamus.

In our second statistical analysis, we also considered XY Sry- and XX Sry+ mice. In particular, two-way ANOVA analyses were performed with gonadal phenotype and sex chromosome complement as two independent variables, followed by post-hoc test (i.e., Tukey's multiple comparison test). As reported in Fig. 1, the statistical analysis performed on PREG plasma levels (panel A) revealed a significant effect of gonadal phenotype (see also Table 2, for statistical details). Two-way ANOVA indicated a significant effect of gonads also in the case of PROG (panel B) and of its metabolites, DHP (panel C), ALLO (Panel D), and ISOALLO (panel E, and see also Table 2, for statistical details). In case of ALLO interaction was also observed, while for ISOALLO levels a significant effect of sex chromosome complement and interaction of the two variables were also reported (see also Table 2, for statistical details). In particular, the post-test indicated that the plasma levels of ALLO (panel D) were significantly lower in XY Sry+ vs. XY Sry- ($p < 0.0001$) and in XX Sry- vs. XY Sry- ($p = 0.0154$) and in XX Sry+ vs. XY Sry- ($p < 0.0001$), while those of the further metabolite ISOALLO (panel E) were higher in XY Sry+ vs. XY Sry- ($p = 0.0025$) and vs. XX Sry+ ($p = 0.0025$).

As reported in Fig. 2, assessment of the plasma levels of DHEA (panel A) did not show statistically significant differences, however, the further metabolites T (panel B), DHT (panel C), and 3α-diol (panel D) were significantly different. Indeed, in the case of T (panel B), two-way ANOVA showed a significant effect of gonads, sex chromosome complement, and interaction of the two variables (see also Table 2, for statistical details). The levels of the further metabolites DHT (panel C) and 3α-diol (panel D) only showed a significant effect of gonads (see also Table 2, for statistical details). In particular, the levels of T were significantly higher in XX Sry+ vs. XX Sry- ($p = 0.0008$), vs. XY Sry- ($p = 0.0018$), and vs. XY Sry+ ($p = 0.0135$).

Fig. 3 shows the hippocampal levels of PREG (panel A), PROG (panel B), DHP (panel C), ALLO (panel D), and ISOALLO (panel E) in the 4 experimental groups considered. Two-way ANOVA reported a significant effect of gonads in the case of PREG, PROG, and DHP (see also Table 2, for statistical details). Interaction was observed in the case of PREG levels. No significant changes were observed in the ALLO (panel D) and ISOALLO (panel E) levels. Post-test revealed that the levels of PREG (panel A) were significantly higher in XY Sry+ vs. XY Sry- ($p = 0.0012$) and vs. XX Sry+ ($p = 0.0084$).

As reported in Fig. 4, a significant effect of the gonads was observed in the hippocampal levels of DHEA (panel A). In agreement with the plasma pattern, T levels (panel B) also in the hippocampus revealed a

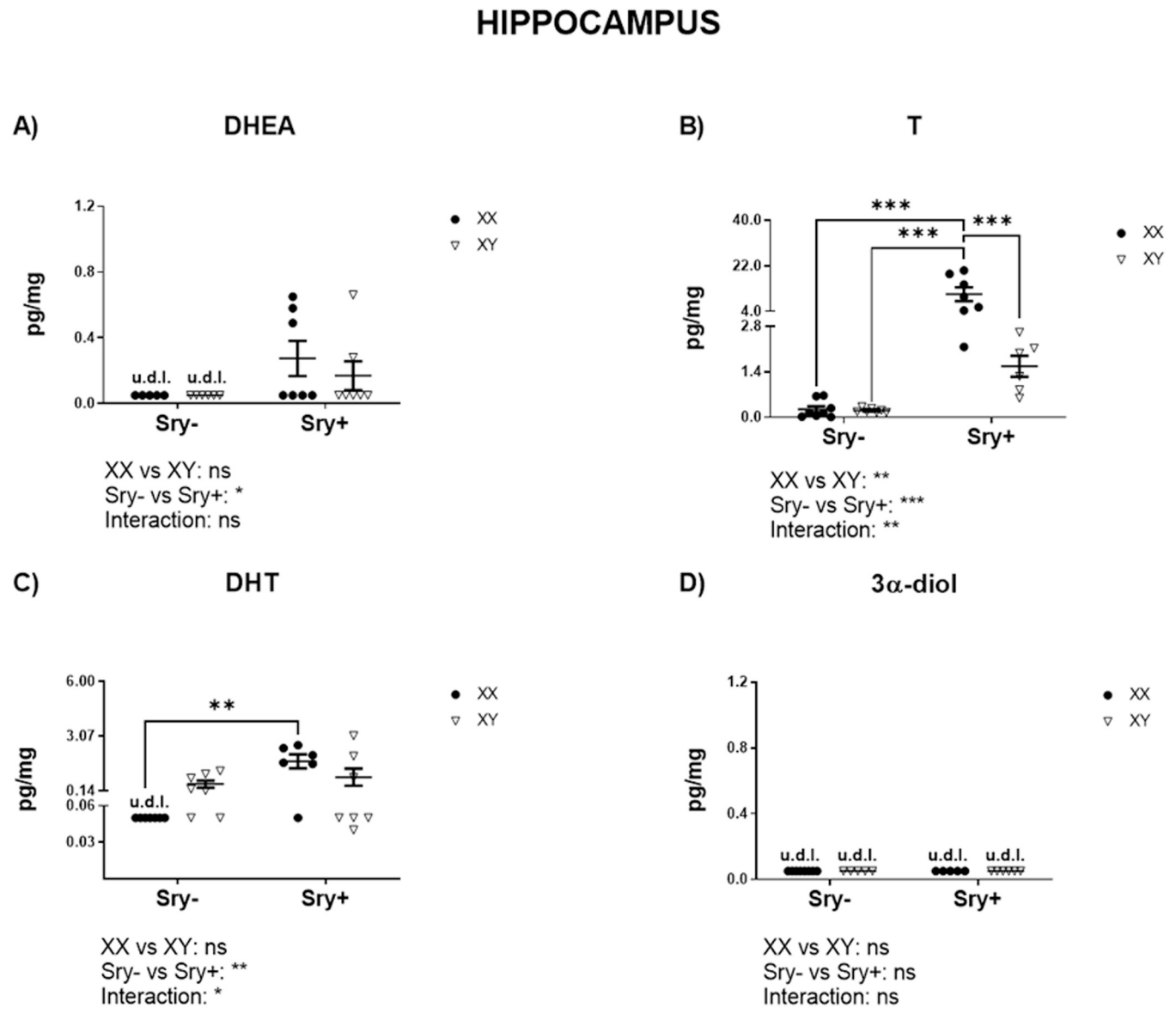


**Fig. 4.** Hippocampal levels assessed by LC-MS/MS of four core genotype animals. The panels represent the levels of (**A**) dehydroepiandrosterone (DHEA); (**B**) testosterone (T); (**C**) dihydrotestosterone (DHT); (**D**) 5α-androstane-3α, 17β-diol (3α-diol). Data are expressed as pg/mg and are the mean ± SEM. u.d.l. = under detection limit. Detection limit was 0.05 pg/mg for DHEA, DHT and 3α-diol. n = 5–8 animals for each experimental group. The two-way ANOVA was used for statistical analysis followed by the multiple comparison Tukey post-hoc test. Statistically significant differences of sex chromosme complement (XX vs XY), gonadal phenotype (Sry- vs Sry+) and the interaction of sex chromosome complement with gonadal phenotype (interaction) are indicated as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns, not significant difference.

significant effect of gonads, sex chromosome complement, and interaction of the two variables (see also Table 2, for statistical details). In addition, the DHT hippocampal levels (Fig. 4, panel C) showed, in agreement with its plasma levels (Fig. 2, panel C), a significant effect of gonads and interaction (see also Table 2, for statistical details). The levels of 3α-diol were unmodified (panel D). Post-test showed that T levels (panel B) were higher in XX Sry+ vs. XX Sry- ($p < 0.0001$), vs. XY Sry- ($p < 0.0001$), and vs. XY Sry+ ($p = 0.0006$). DHT levels (panel C) were higher in XX Sry+ vs. XX Sry- ($p = 0.0050$).

Fig. 5 shows the hypothalamic levels of PREG (panel A), PROG (panel B), DHP (panel C), ALLO (panel D) and ISOALLO (panel E) in the 4 experimental groups considered. Two-way ANOVA reported a significant effect only of gonads in the case of PROG (see also Table 2, for statistical details) and of gonads and sex chromosome ($F_{1,16} = 5.389$ and $p = 0.0388$) in the case of ALLO (see also Table 2, for statistical details). As reported in plasma, also in the hypothalamus, the levels of DHEA were unmodified in the four experimental groups considered (Fig. 6, panel A). Two-way ANOVA reported a significant effect of gonads on hypothalamic levels of T (panel B), DHT (panel C), and 3α-diol (panel D, see also Table 2, for statistical details). A significant effect of the sex chromosome and interaction was also reported in the case of T and 3α-diol (see also Table 2, for statistical details). Post-test indicated that the levels of T were higher in XX Sry+ vs. XX Sry- ($p < 0.0001$), vs. XY Sry- ($p < 0.0001$), and vs. XY Sry+ ($p = 0.0013$). The levels of 3α-diol were higher in XY Sry+ vs. XY Sry- ($p = 0.0408$) and vs. XX Sry+ ($p = 0.0408$).

## 5. Discussion

Our findings show that neuroactive steroid levels are influenced by a combination of gonadal and sex chromosome effects that is specific to each steroid and each analyzed compartment. These results are in

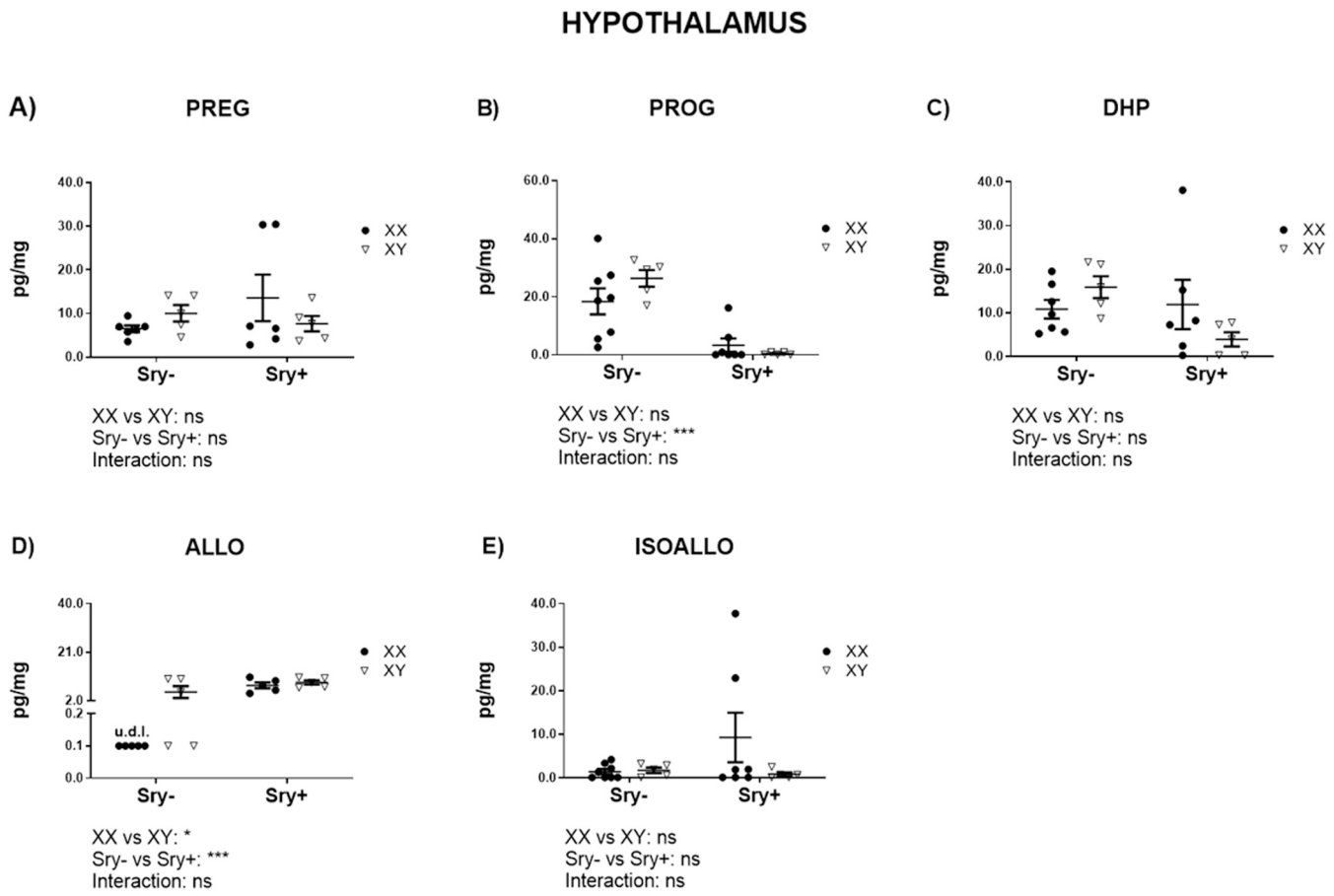


**Fig. 5.** Hypothalamic levels assessed by LC-MS/MS of four core genotype animals. The panels represent the levels of (**A**) pregnenolone (PREG); (**B**) progesterone (PROG); (**C**) dihydroprogesterone (DHP); (**D**) allopregnanolone (ALLO); (**E**) isoallopregnanolone (ISOALLO). Data are expressed as pg/mg and are the mean ± SEM. u.d.l. = under detection limit. Detection limit was 0.1 pg/mg for ALLO. n = 5–8 animals for each experimental group. The two-way ANOVA was used for statistical analysis followed by the multiple comparison Tukey post-hoc test. Statistically significant differences of sex chromosme complement (XX vs XY), gonadal phenotype (Sry- vs Sry+) and the interaction of sex chromosome complement with gonadal phenotype (interaction) are indicated as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns, not significant difference.

agreement with previous observations suggesting that extragonadal factors affect the local synthesis, metabolism, or transport of neuroactive steroids in different brain regions [4,12]. Thus, changes in neurosteroid levels after castration are not identical in the brain and plasma and are specific for each brain region [3]. In addition, previous studies in rats [11] and our present findings in mice indicate that there is not a correspondence between sex differences in neurosteroid levels in plasma, the hippocampus, and the hypothalamus, further supporting that there is not a strict correlation between the central and circulating levels of neuroactive steroids [11,13].

As expected, our results in the FCG mouse model showed that the gonadal phenotype had a strong influence on the levels of neuroactive steroids, being the unique factor that determines sex differences in PROG levels in plasma and the brain, PREG and 3α-diol levels in plasma, and DHP, ALLO and DHT levels in hypothalamus. In addition, gonadal phenotype significantly affected the levels of T in all compartments, ALLO and ISOALLO in plasma, PREG and DHT in hippocampus and 3α-diol in hypothalamus.

However, the gonadal phenotype was not the exclusive determinant of neuroactive steroid levels, because the sex chromosome complement exerted a significant effect *per se* and showed a significant interaction with the gonadal phenotype in the levels of T in all compartments examined.

For other steroids, the effect of sex chromosome complement and its interaction with gonadal effects were specific to each analyzed compartment. Thus, the sex chromosome complement had a significant interaction with gonads in determining the levels of ALLO and ISOALLO in plasma, the levels of PREG and DHT in hippocampus and the levels of 3α-diol in the hypothalamus, exerting also a significant effect in the levels of ALLO in the hypothalamus.

These effects of sex chromosome complement on neuroactive steroid levels could be explained by the escape tissue-specific X chromosome inactivation observed for numerous X chromosome genes [14]. Many of these X chromosome genes that escape inactivation are transcription regulators. One example is *Kdm6a/Utx*, which encodes a demethylase involved in chromatin remodeling and neuronal phenotypic differentiation [15,16]. Among numerous other physiological events, this gene regulates cholesterol synthesis, steroidogenesis [17], and sex steroid receptor signaling [18]. Therefore, it is a potential generator of sex differences in steroidogenesis and in the regulation exerted by gonadal steroids on the expression of steroidogenic enzymes in the brain. Other X-linked genes that can affect steroid levels are those that encode for proteins that regulate steroidogenesis, such as dosage-sensitive sex reversal-adrenal hypoplasia congenital on the X-chromosome gene-1 (*Dax-1)* [19,20], or steroid metabolism, such as steryl-sulfatase [21].

In comparison with autosomes, the X chromosome is relatively enriched in neural genes, which are expressed at different levels in male and female brains [22–30]. Some of these genes, together with a few Y-linked genes that also show sex-dimorphic expression in the brain [31–33], may participate in the observed effects of sex chromosome complement on neuroactive steroid levels in the hippocampus and hypothalamus by generating regional sex differences in steroid synthesis or

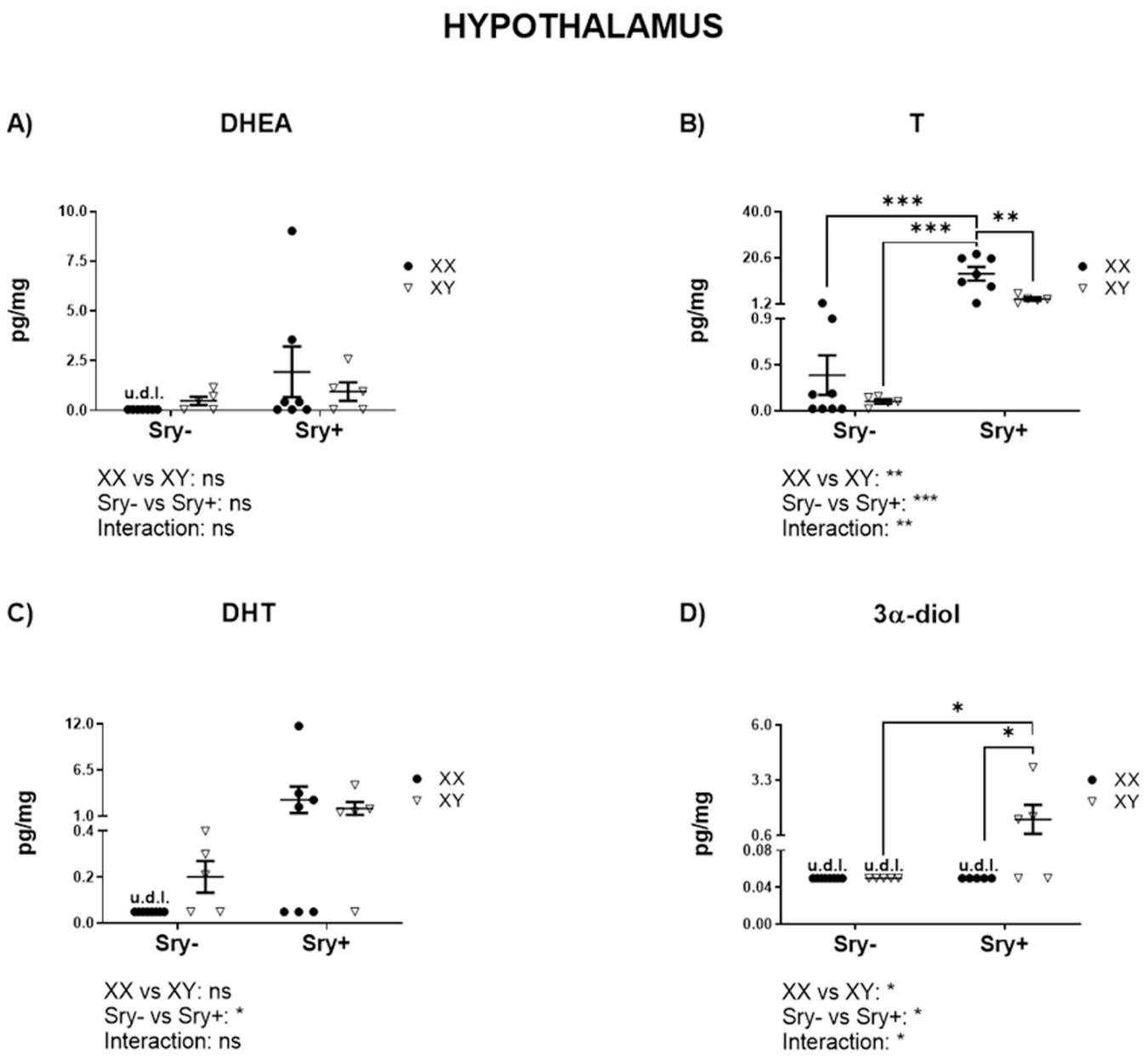


**Fig. 6.** Hypothalamic levels assessed by LC-MS/MS of four core genotype animals. The panels represent the levels of (**A**) dehydroepiandrosterone (DHEA); (**B**) testosterone (T); (**C**) dihydrotestosterone (DHT); (**D**) 5α-androstane-3α, 17β-diol (3α-diol). Data are expressed as pg/mg and are the mean ± SEM. u.d.l. = under detection limit. Detection limit was 0.05 pg/mg for DHEA, DHT and 3α-diol. n = 5–8 animals for each experimental group. The two-way ANOVA was used for statistical analysis followed by the multiple comparison Tukey post-hoc test. Statistically significant differences of sex chromosme complement (XX vs XY), gonadal phenotype (Sry- vs Sry+) and the interaction of sex chromosome complement with gonadal phenotype (interaction) are indicated as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns, not significant difference.

metabolism.

It should be noted that the interaction of gonadal factors and sex chromosome complement in the regulation of neuroactive steroid levels could be bidirectional because gonadal hormones cause changes in chromatin accessibility and may therefore regulate the expression of sex chromosome genes [34]. Furthermore, gonadal hormones and sex chromosome genes could cooperate in increasing sex differences in neuroactive steroid levels or can exert a mutual compensation of their effects. This latter possibility is suggested by the intriguing observation that the XX males had higher plasma, hippocampal, and hypothalamic levels of T than the XY males. Thus, while the presence of testes promotes higher testosterone levels compared with females, the XY genotype appears to reduce the magnitude of this sex difference.

In contrast, our findings suggest that sex differences in the levels of other steroids result from synergistic interactions between the effects of gonads and sex chromosomes. This is the case of ISOALLO, whose plasma levels are determined by the addition of gonadal and genotype effects. Indeed, XY animals with testes showed significantly higher plasma ISOALLO levels than the other animal groups. Therefore, the combination of the XY genotype with the presence of the testis is necessary to generate a sex difference in the plasma levels of this neuroactive steroid.

In conclusion, our findings suggest that tissue-specific actions of sex chromosome genes interact with gonadal secretions in the determination of neuroactive steroid levels in plasma and the brain. This conclusion has practical implications, because numerous neurological and psychiatric disorders are associated with sex- and regional-specific alterations in the levels of neuroactive steroids [12]. Thus, considering the

accumulative number of new observations that are detecting the implication of sex chromosome genes on pathological conditions of the nervous system [29,35–44], it will be essential to explore the potential link between sex chromosome genes, alterations in neurosteroid levels, and brain pathology in future studies. For instance, the identification of an influence of sex chromosome genes on the levels of steroids that are altered in bipolar disorder and contribute to maniac episodes [45] may potentially result in the identification of a new avenue for therapeutic interventions. Similar considerations apply for traumatic brain injury [46], stroke [47], Parkinson's disease [48,49], Alzheimer's disease [50], and other neurodegenerative conditions in which brain or plasma changes in neuroactive steroid levels are associated with the amount of neural damage or the severity of clinical manifestations.

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## CRediT authorship contribution statement

**Roberto Cosimo Melcangi:** Writing – original draft, Conceptualization. **Silvia Giatti:** Writing – review & editing, Funding acquisition, Conceptualization. **Maria-Angeles Arevalo:** Supervision, Investigation. **Luis Miguel Garcia-Segura:** Writing – original draft. **Donatella Caruso:** Writing – review & editing, Supervision. **Daniel Pinto-Benito:** Investigation. **Daniela Grassi:** Writing – review & editing, Formal analysis, Conceptualization. **Silvia Diviccaro:** Writing – review & editing. **Lucia Cioffi:** Writing – review & editing, Formal analysis, Data curation.

## Declaration of Competing Interest

The authors report no declarations of interest

## Data Availability

Data will be made available on request.

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